Biofilms and Wound Infection Research in the US Military

Kevin S. Akers, Joseph C. Wenke, and Clinton K. Murray

Abstract Recent US military conflicts have involved severe extremity injuries frequently requiring implantation of orthopedic stabilizing devices. Simultaneously, bacterial wound contamination, including by multidrug-resistant organisms, has presented a significant clinical challenge due to reduced antimicrobial treatment options, with an unclear but likely contribution from biofilm formation on implanted devices. In this chapter, we detail investigations conducted by the US military medical research community into wound infections occurring in casualties from conflicts in Iraq and Afghanistan.

Keywords Military · Conflicts · Wound · Biofilm · Antimicrobial · Research

Introduction

US military combat operations in Iraq (Operation Iraqi Freedom, OIF) and Afghanistan (Operation Enduring Freedom, OEF) frequently involved a high number of extremity injuries. This is likely multifactorial, influenced by the use of body armor protecting the trunk but not the extremities, tourniquets permitting survival to surgical care by controlling potentially lethal extremity hemorrhage, and enemy tactics involving the use of improvised explosive devices (IEDs). In Afghanistan, IEDs were frequently buried underground and detonated when military personnel

K. S. Akers (✉)
United States Army Institute of Surgical Research, Fort Sam Houston, TX, USA

Uniformed Services University of the Health Sciences, Bethesda, MD, USA
e-mail: Kevin.s.akers.mil@mail.mil

J. C. Wenke
United States Army Institute of Surgical Research, Fort Sam Houston, TX, USA

C. K. Murray
Uniformed Services University of the Health Sciences, Bethesda, MD, USA

1st Area Medical Laboratory, Aberdeen Proving Ground, Aberdeen, MD, USA

D. L. Williams (ed.), *Targeting Biofilms in Translational Research, Device Development, and Industrial Sectors*,
https://doi.org/10.1007/978-3-030-30667-0_4

on foot (known as a dismounted patrol) were in the vicinity or overtop of the ordnance, resulting in an injury pattern which came to be known as a "dismounted complex blast injury." Such injuries generally involved the lower extremities and possibly the groin and were frequently characterized by extensive soft tissue damage, vascular injury, and complex fractures requiring hardware stabilization and definitive fixation to achieve fracture healing. A further complication is that both trauma and transfusion are significantly immunomodulatory, with little known about the impact on clinical outcome or susceptibility to infection [1].

Contemporaneously, bacterial pathogens with broad antimicrobial resistance became established in US military facilities due to a convergence of factors: (1) a growing global pandemic of bacteria harboring various mechanisms of resistance to antimicrobials, known as multidrug-resistant organisms (MDROs); (2) care provided to local civilians, representing a source of MDROs entering deployed military medical facilities [2]; and (3) challenges with adherence to infection control measures in these facilities, particularly during mass casualty events and intercontinental air transport of combat casualties [3]. The resulting clinical circumstance of severe complex traumatic extremity wounds requiring indwelling hardware with possible contamination by MDRO organisms sets the stage for a multiyear outbreak of combat-related extremity wound infection in the US military healthcare system [4]. Although clinical confirmation was, and remains, limited by a lack of available diagnostic tests, bacterial biofilms colonizing implanted orthopedic fixation devices may further complicate the care of these patients by leading to persistent or relapsing infections [5].

Clinical Impact of Extremity Injury in US Military Combat Casualties

The impact of combat-related extremity injuries on US military personnel from OIF/OEF was documented in a series of studies performed at the US Army Institute of Surgical Research and several military treatment facilities in the United States. Extremity injuries were highly prevalent, with approximately 82% of combat casualties having at least one extremity injury [6]. Overall, 24–27% of extremity injuries developed infection, with 17% of those experiencing recurrence [7, 8]. The treatment of extremity wound infection typically involves multiple debridement surgeries followed by antibiotic therapy for weeks to months. Wound dehiscence was a major problem in the treatment of combat wounds, with wound debridement for infection being the most common procedure in battlefield injured service members. Approximately 12% of casualties required hospital readmission for wound infection debridement [9]. This in turn was a leading cost driver of medical care for US military combat casualties.

Not unexpectedly, the wound infection problem has been a major detriment to combat casualties with lower extremity orthopedic injuries, leading to increased

amputation rates and decreased return-to-duty rates. Among 115 military casualties with type III open tibial fractures sustained between 2003 and 2007, the return-to-duty rate was just 18% if tibial fracture was the only injury, decreasing to 12.5% if an amputation was performed [10]. Illustrating the impact of infection, 92% of those with osteomyelitis were medically retired from military service. Sixty-nine percent were unfit for service due to a tibial fracture, and those with amputations received a higher Veteran's Affairs (VA) disability rating. A second study of military casualties receiving amputations between 2001 and 2006 found return-to-duty rates of 18–22% for tibial or femoral amputations, and 17% for humeral or radial amputations [11]. In a cohort of 115 soldiers with a type III open tibial fracture, 40% suffered an infectious complication and 94/115 (81.7%) were unable to return to duty and were medically retired [12]. In a study of late amputations after combat-related type III open tibial fractures, patients receiving late amputations had the highest rates of deep soft tissue infection (72.7%) and osteomyelitis (54.5%). Those who had late amputations had higher rates of grafting with autologous bone or bone morphogenic protein (BMP) combined than those with successful limb salvage (27.3% vs 4.8%, $p < 0.01$) [13].

Clinical Impact of Trauma-Related Infection in US Military Combat Casualties

The Trauma Infectious Disease Outcomes Study (TIDOS) [14] prospectively collected injury and infection data from combat casualties from Iraq and Afghanistan between 2009 and 2015. While analysis of these data is ongoing, a preliminary report has summarized the first 3 years of data (2009–2012) [15]. Over this period, 1807 combat casualties were included in the study, with 34% having an infection. Of those with infections, 57% had more than one infection. Independent risk factors for infection included amputation, blood transfusion within 24 hours of injury, ICU admission at Landstuhl Regional Medical Center (LRMC), severe or life-threatening Injury Severity Score, and mechanical ventilation.

Johnson and colleagues at the Brooke Army Medical Center Infectious Disease Service reported on infectious outcomes of patients with Gustilo-Anderson type III open tibial fractures (11 type IIIa and 24 type IIIb) in US military personnel occurring in Iraq and Afghanistan between 2003 and 2006 [16]. Seventy-seven percent were associated with an explosive device as the mechanism of injury. Casualties were managed initially with perioperative debridement including use of Gram-positive coverage with vancomycin or cefazolin. They were transported to LRMC in Germany, where a single debridement surgery was typically performed prior to transport back to the United States for definitive medical care, on average 7.4 days after injury. Twenty-seven (77%) had positive initial cultures at the time of admission to a US hospital. The most commonly recovered organisms in culture were *A. baumannii-calcoaceticus* complex, *Enterobacter* spp., and *P. aeruginosa*.

Polymicrobial infection was documented in 10 cases (37%). Most patients (89%) were treated for osteomyelitis, with only three treated for deep wound infection. Thirteen cases (37%) were complicated by a recurrence of deep wound infection involving the fracture site, of which 11 had previous infections. Most notably, almost all the initial infections involved Gram-negative organisms, whereas all of the recurrent infections involved *S. aureus*. Additionally, culture-positive infection was a contributing factor to four of five amputations. The fifth amputation was associated with tibial non-union without culture-proven evidence of infection [16].

Following this report, Yun and colleagues from the same institution examined cases of osteomyelitis (not restricted to tibial fractures) in military personnel injured in Iraq and Afghanistan over approximately the same time period [17]. They analyzed 101 patients who experienced 103 initial and 36 subsequent hospitalizations, with 94 involving lower extremities, 43 involving upper extremities, and 2 involving the axial skeleton. Recurrent infection occurred in 19 patients (18%). While Injury Severity Score was not found to be different between patients with non-recurrent and recurrent infections, Gram-negative organisms were more likely to occur in the initial infection, particularly involving *A. baumannii-calcoaceticus* complex, *Klebsiella pneumoniae*, and *P. aeruginosa.* Similar to Johnson's report [16], recurrences were associated with recovery of Gram-positive organisms, particularly *S. aureus* and coagulase-negative staphylococci, and less likely to be polymicrobial. Suggesting a contribution from biofilms in recurrent infections, the presence of an internal fixation device was the only factor found to be associated with recurrence of infection in a univariate analysis. Ninety percent of these patients received antimicrobial therapy for greater than 4 weeks, and 78% were treated longer than 6 weeks. An important finding from this analysis was that among patients with recurrence involving methicillin-resistant *S. aureus* (MRSA) in whom it was not present in the initial infection, 67% had received vancomycin for greater than 2 weeks. This finding highlights the important role of antimicrobial stewardship in the treatment of such patients and argues against the presumptive or prophylactic use of vancomycin without an appropriate microbiological indication [17].

Data from the Department of Defense Trauma Registry (previously called the Joint Theater Trauma Registry) [18] enabled a case-control study to determine risk factors for osteomyelitis among combat casualties with open tibial fractures occurring between 2003 and 2009 [19]. One-hundred thirty cases (patients with infected open tibial fractures) were compared to 85 controls (patients with open tibial fractures without infection at that site). The Gustilo-Anderson fracture classification was modified to include trans-tibial amputation (TTA) as the most severe grade. Excluding TTA, cases required longer time to achieve radiographic union (median, 210 versus 165 days). Risk factors for osteomyelitis included blast mechanism of injury, utilization of antibiotic beads, Gustilo-Anderson grade greater than IIIb, and presence of foreign bodies at the fracture site. Notably, TTA carried the highest risk of infection. A separate analysis utilizing the Orthopedic Trauma Association Open Fracture Classification system demonstrated a spectrum of increasing infection risk associated from muscle loss to muscle death [19].

The TIDOS data support a very important and unique longitudinal collaborative effort with the Department of Veterans' Affairs (VA) hospital system [20]. The

purpose is to document long-term infection risk and outcomes among combat casualties after being discharged from the military after which time their medical care often transitions to the VA system. Although work is still ongoing to fully characterize the cohort, an initial publication documents the findings from the initial 337 for whom complete data have been abstracted and analyzed. One-hundred eleven (33%) had at least one infection related to their traumatic injury during hospitalization in the US Department of Defense medical system (totaling 244 unique infections). The most common were skin/soft tissue infection (SSTI, 43%) and osteomyelitis (14%). After the initial hospitalization, 127 patients (38%) developed 239 new infections related to their traumatic injury. Twenty-nine percent of these infections occurred after leaving military service. Independent risk factors for reduced time to infection following initial hospitalization were more severe injuries (Injury Severity Score > 10) and having an infection during the initial hospitalization. Notable findings within the VA phase of care were a "second peak" of SSTI and osteomyelitis 6–12 months after the initial infection and more frequent diagnosis of urinary tract infections, potentially reflecting perineal trauma from dismounted complex blast injury [20].

The Role of Biofilms in Extremity Wound Infection

Many combat-related extremity injuries are complex, involving extensive bone and soft tissue damage and requiring the presence of artificial materials to maintain the spatial orientation and anatomic alignment of bone fragments necessary to achieve an acceptable result of healing [21]. An unintended consequence of hardware required to stabilize mangled extremities is that they may serve as a substrate for bacterial attachment and the development of biofilms. Biofilms can develop when bacteria expand horizontally and vertically on a solid surface, forming a sessile, multicellular colony which secretes a matrix of protein, polysaccharide, and extracellular DNA that impedes the penetration of antimicrobials [22]. Bacteria in the lower strata of the colony cease replication and thus become tolerant to currently approved antimicrobials and create persister cells. In addition, the degree of soft tissue damage and vascular disruption in these extensive wounds likely limits antimicrobial penetration to site of infection. As a result, systemic treatment regimens with currently approved antimicrobial agents cannot predictably eradicate biofilms [23], which therefore pose a risk for infectious relapse in the setting of devices that must be retained for healing. Thus, adequate surgical debridement is required to mitigate these infections. A limitation to this approach is that there is no objective way for surgeons to judge when debridement has been sufficient and final wound closure can be performed with a minimal risk of infectious relapse. Complicating and adding to this, the soft tissue injuries are so severe that the wounds cannot be closed immediately, often being debrided in different operating rooms while the patient is being evacuated from the Middle East to Germany, and eventually arriving at military hospitals in the United States. Unfortunately, the patient records are often incomplete and may not accompany the Wounded Warrior, leading to a common practice of writing last time of debridement and treatment of wound on the exterior

of dressings with a marker. Thus, a biomarker that could be used to accurately and objectively predict successful wound closure would be highly advantageous.

Studies by US military-affiliated investigators have included both natural history and basic science approaches. Investigators at the Walter Reed National Naval Medical Center and nearby Uniformed Services University of the Health Sciences in Bethesda, Maryland, evaluated the wound effluent captured from negative-pressure wound therapy for biomarkers useful to predict wound dehiscence. Forsberg and colleagues found an association between non-healing wounds and decreased effluent levels of RANTES and IL-13 [24]. Hawksworth and colleagues then examined other inflammatory biomarkers in serum and effluent, finding that increased serum IL-6, IL-8, and MIP-1α predicted wound healing. In the effluent, increased IL-6 and decreased IL-2 and IP-10 predicted healing [25]. Utz and colleagues examined metalloproteinases (MMP), finding that increased serum MMP-2 and MMP-7, and decreased effluent MMP-3, predicted impaired healing [26]. Brown and colleagues examined the microbial burden in combat wounds, defining a threshold of critical bacterial colonization of the wound as $>10^5$ CFU/g above which wound dehiscence is more likely to occur. Dehiscence was predicted by increased serum IL-6, -8, -10, MIP-1α, MMP-7, and MMP-13, as well as increased IL-6, IL-8, and MIP-1α in wound effluent [27]. Evans and colleagues examined the relationship of critical bacterial colonization (here defined as $>10^5$ CFU/cm^3) to heterotopic ossification (HO), finding that increased IL-6, IL-10, and MCP-1 in serum, and increased MIP-1α and decreased IP-10 in effluent, predicted development of HO [28].

Assessments of the clinical impact of biofilm are limited. At the US Army Institute of Surgical Research, Sanchez et al. investigated the biofilm production by 205 clinical strains of bacteria obtained from various solid and liquid source tissues of 150 patients with relapsing infections [29]. Among these isolates from wound, bone, respiratory, urinary tract, and blood isolates, later-recovered isolates were identical clones (by pulsed-field gel electrophoresis) of initially recovered strains. Biofilm formation, characterized by crystal violet staining of 48-hour growth in vitro, was heterogeneous, with increased biofilm production among isolates recovered from non-liquid sources.

Using prospectively collected data from the Trauma Infectious Disease Outcomes Study (TIDOS) [14], we examined risk factors including for the persistence of clinical wound infections meeting CDC/NHSN criteria for skin and soft tissue infection [5]. This study defined "persistence" as recovery of clonally identical isolates at least 14 days apart. Thirty-five persistently infected wounds from 25 patients were compared to 69 wounds from 60 patients with a single episode of wound infection. We identified biofilm formation to be a univariate risk factor (OR 29.49, 95% CI 6.24–∞) but lacked sufficient clinical data to associate this finding with implanted medical devices. Further, univariate risk factors also included MDRO phenotype, packed red blood cell transfusion within the first 24 hours after injury, number of operating room visits prior to and on the date of infection diagnosis, anatomic location of infection, and polymicrobial infection. Independence of these risk factors for prediction of infection persistence unfortunately could not be established, as the

small sample size prevented convergence of a multivariate logistic regression model. *Acinetobacter baumannii* was the only species for which higher biofilm formation was statistically associated with wound infection persistence. This study suggests a possible linkage between biofilm formation as a phenotypic trait and persistent wound infection.

Heitcamp and colleagues utilized the TIDOS database to explore the role of *Enterococcus* spp. in clinical infection, as it was among the most frequent clinically isolated organisms in this data set [30]. Using a case-comparator study design, Heitcamp and colleagues compared 155 cases having *Enterococcus* spp. recovered within 3 days of wound infection diagnosis to 237 comparators for whom other organisms (but not *Enterococcus* spp.) were recovered in this time frame. *E. faecium* was the most common species (65.7%) followed by *E. faecalis* (12.5%), with most isolates arising in the setting of multiple and polymicrobial infections. Case patients were more likely to have a higher injury severity score and an increased rate of ICU admission and have received more transfused units of packed red blood cells and/or whole blood within 24 hours of injury, more operating room visits, and a longer length of hospitalization. Although biofilms were not specifically addressed in this manuscript, biofilm characterization of TIDOS *Enterococcus* isolates performed at the US Army Institute of Surgical Research showed almost universally low biofilm formation (unpublished data). An ensuing collegial debate about this finding among members of the TIDOS investigative team highlighted the question of what is the most appropriate manner for growth and characterization of biofilm-forming potential of clinical isolates. We previously explored this issue in the laboratory using clinical and reference strains of *S. aureus*, characterizing biofilm growth without human plasma, or with various concentrations of plasma either coating the growth plate, or added to the liquid growth medium. We found *S. aureus* to be optimally stimulated by addition of 10% human plasma to growth medium, increasing the crystal violet uptake signal by between 2.5- and nearly ten-fold depending on the strain. This was accompanied by dramatic fold changes in the expression of genes regulating matrix adhesion molecules (MSCRAMMs) between 30 and 120 minutes, with some increasing and others decreasing [31]. As growth conditions reported in biofilm literature vary widely (which we have previously reviewed [32]), and the biofilm production of some organisms is known to be significantly impacted by host factors likely to be present in wounds [31], a consensus method is currently lacking. Harmonization of biofilm testing conditions for clinical strains which accurately recapitulates in vivo biofilm-productive behavior in clinical infections would be a significant advance for the field.

Strategies for Biofilm Mitigation

Research efforts within the US military medical research enterprise have targeted potential approaches by which to mitigate biofilm formation in wounds. At the US Army Institute of Surgical Research, Wenke and colleagues established the rat

femur segmental bone defect model to conduct research on various aspects of orthopedic trauma [33]. This model attempts to recapitulate the time course of care provided for human long-bone fractures, with delayed presentation to treatment, followed by irrigation, debridement, and long-bone stabilization by fixation with implanted materials. The carefully controlled addition of a bacterial inoculum to establish what is likely a biofilm-mediated orthopedic device infection makes this model a useful platform by which to explore and evaluate optimal approaches to mitigate biofilm formation. Favorable results can then be "scaled up" into larger animal models. Studies to date (some using goats) have characterized optimal approaches to fluid lavage of contaminated wounds [34], various antiseptic additives [35] and chlorhexidine [33], and Dakin's solution (buffered hypochlorite) as well as proprietary substances. A unifying theme which has emerged from this work is that local tissue damage within the wound, whether chemical or mechanical/physical, promotes bacterial growth in spite of initial reductions in bacterial burden. Therefore, an ideal substance for topically applied prophylaxis or treatment of wound infections would be one that is rapidly lethal to pathogens but innocuous to host mammalian tissues. Whereas conventional toxicity testing, driven by regulatory requirements for the cosmetic and topical medication industries, relies on skin cells such as keratinocytes and fibroblasts, we have noted that cell types relevant to deeper wound and bone tissues (such as myocytes, osteoblasts, etc.) seem to be more vulnerable to toxic insult [36–38]. Importantly, the regulatory approach for topical antiseptics has relied on toxicity testing using intact skin as opposed to deeper tissues which become exposed and contaminated in severe blast injuries or high-velocity penetrating trauma.

In addition to topical antiseptics, we have examined several conventional systemic antibiotics for potential repurposing as topical antimicrobials applied to contaminated wounds. This concept evolved from clinical reports indicating a decrease in infection rates associated with human spine surgery when vancomycin powder was topically applied as a prophylactic within clean surgical wounds [39–41]. Vancomycin powder and polymethyl methacrylate (PMMA) beads impregnated with 10% vancomycin (wt/wt) were examined in the rat femoral segmental defect model inoculated with *Staphylococcus aureus* UAMS-1. Animals received debridement and vancomycin powder or vancomycin-impregnated PMMA bead placement either 6 or 24 hours after contamination [42]. Significant reductions in bacterial burden were observed when either treatment was applied 6 hours after inoculation but failed to prevent wound infection when applied 24 hours after inoculation. This is postulated to represent the effect of biofilm maturation on the implanted orthopedic stabilizing device over this time period. Thus, the time elapsed from a contaminating event until debridement may constitute an important factor influencing the outcome of wound infection. Early irrigation was found beneficial in a goat contaminated wound model, perhaps denying an opportunity for biofilms to become established [43]. In humans, longer times to debridement of open tibial/fibular fractures (a condition known to be high risk for infection) were correlated with increased rates of clinical infection [44]. Another unifying theme has been that if there is a substantial time delay from time of contamination to treatment, an approach that addresses biofilms is needed.

Rifampin has a unique role among antimicrobial agents currently approved for use in humans, as it is considered the most suitable agent for the treatment of bacterial biofilms. Rifampin is less soluble in water than most other human-use antibiotics and has been demonstrated in the laboratory to have an increased capacity for physical penetration into biofilms [45]. Despite relatively limited human clinical data, the use of systemic rifampin in combination with other antimicrobials is recommended for prosthetic valve endocarditis and osteoarticular infections involving prosthetic devices (thought to involve biofilms) in guidelines for the treatment of methicillin-resistant *S. aureus* (MRSA) infections by the Infectious Disease Society of America [46].

We explored in our laboratory the potential utility of topically applied rifampin delivered in PMMA beads [47]. A common practice in orthopedic surgery, PMMA is polymerized from two components in an exothermic chemical reaction and mixed at the time of use, curing to a hard, rigid material within 15 minutes. Heat-stable antibiotic powders such as vancomycin, gentamicin, or tobramycin can be added at the surgeon's discretion. Using the rat contaminated femoral segmental defect model with 6- or 24-hour post-contamination debridement, Shiels et al. demonstrated that 10% wt/wt rifampin in PMMA significantly reduced colony-forming units of *S. aureus* UAMS-1. This was in contrast to lower rifampin loading masses of 1% (wt/wt), rifampin 1% with vancomycin 1.7%, or rifampin 1% with vancomycin 5%. The 10% rifampin-loaded PMMA beads demonstrated burst-release elution kinetics with continued elution over 13 days. Notably, however, this amount of rifampin resulted in incomplete curing of the PMMA, resulting in beads which took between 1 and 2 hours to cure to a final elastic state. In vivo, only 10% rifampin beads prevented bacterial growth on the implants and the PMMA beads themselves, in contrast to the other formulations tested. Recovery of bacterial growth from some of the PMMA beads suggests that this approach can perpetuate infection by providing a surface for bacterial growth. In contrast to the other treatment groups, animals treated with 10% rifampin-loaded PMMA had no signs of clinical infection and significantly lower 14-day bacterial counts from bone, hardware, and tissue when debrided after 6 hours. Delaying debridement for 24 hours worsened these results overall, but nevertheless bone, orthopedic hardware, and tissue appeared to become sterilized in some animals at the 14-day end point. This again suggests an important role for early intervention in contaminated orthopedic trauma to prevent infections, possibly reflecting the role of bacterial biofilms in creating tolerance to antimicrobials. Importantly, a screen for phenotypic rifampin resistance greater than 4 μg/mL found none among recovered isolates. Other delivery methods, to include direct application of antibiotic powder, are being explored. For example, topical placement of vancomycin powder is being used to prevent surgical site infections in many orthopedic surgeries; initial reports demonstrate that deep surgical site infection rate of posterior lateral fusions decreases from 2.6% to 0.2% [41].

Prevention of surgical site infections is very different from treating a biofilm infection. Using a rat open fracture model, the time from bacterial contamination to treatment with topical vancomycin powder had a profound effect on the infection. When antibiotic powder is placed 6 hours after contaminating wounds with

S. aureus, none of the wounds had bacteria over the threshold for infection; however, all of the wounds had high levels of bacteria when treatment was delayed to 24 hours. This was expected given the poor performance of vancomycin against biofilms. Unlike vancomycin, topical placement of rifampin powder was successful with both early and delayed treatment in this model [48]. This approach overcomes the issues of poor release kinetics and incomplete curing using bone cements. Although effective against biofilm-based infections, there were concerns that placement of rifampin powder would delay fracture healing because it is one of the most cytotoxic antibiotics to osteoblasts [49]. Follow-up studies demonstrated that rifampin powder placed in wounds does not negatively affect normal bone healing in a rat segmental defect model [50].

A novel approach to biofilm defeat involves utilizing endogenous chemical signals of biofilms to disperse the colony. Various substances have been reported to disperse mature biofilms [51–53]. We examined the biofilm dispersal and prevention properties of norspermidine, a polyamine compound, on MDRO strains of bacteria (*A. baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*) from human clinical infections [54]. We found this substance to have a variable strain- and species-dependent effect on biofilm reduction, with the most pronounced effect on *A. baumannii* by inhibiting motility and reducing the expression of genes encoding quorum-sensing inhibitors. We also observed evidence of significant toxicity in cell cultures and whole tissue explants on histopathology, which may limit consideration of this compound for clinical development. Sanchez et al. utilized a combination of selected D-amino acids (D-Met, D-Phe, D-Trp) as a dispersal agent for biofilms grown from clinical wound strains of *S. aureus* and *P. aeruginosa* [55]. Application of this substance was found to enhance the antibiofilm activity of some currently available antimicrobials, including clindamycin, rifampin, and vancomycin (against *S. aureus* biofilms) and ciprofloxacin and colistin (against *P. aeruginosa* biofilms).

To address the clinical problem of bacterial contamination of synthetic materials necessary for healing critical bone defects (defined as defects that will not heal spontaneously over the lifetime of the organism), Sanchez et al. impregnated D-amino acids into a synthetic polyurethane bone graft [56]. An equimolar mixture of D-Met, D-Pro, and D-Trp significantly reduced bacterial contamination on the scaffold surface in vitro. When implanted in the contaminated femoral segmental defect model, counts of the strong biofilm-former *S. aureus* UAMS-1 were significantly reduced in bone. This serves as a proof-of-concept which suggests that such novel approaches could have utility in the prevention and treatment of clinical infections.

Leveraging its expertise in drug discovery and development, the Walter Reed Army Institute of Research (WRAIR) has contributed to an understanding of combat-related wound infections and explored novel approaches to therapy. Through a collaboration with the Lawrence Livermore National Laboratory, investigators comprehensively evaluated the wound microbiomes of combat casualties using a culture-independent approach based on a massive gene array capable of detecting more than 3500 organisms known to cause infection in vertebrates, as well as a

deep-sequencing approach [57]. The most commonly observed microorganism in the human wound samples was *A. baumannii*, found in 23% of samples. Additionally, presence of the pRAY plasmid of *A. baumannii* was significantly associated with the failure of wounds to heal. *Pseudomonas* spp. were detected more frequently in wounds which failed to heal. Interestingly, an inverse association (favoring wound healing) was found with the presence of organisms associated with the gastrointestinal tract [57]. As part of a broader program investigating the utility of phage (virus-like particles which can infect and kill bacteria) for treatment of antibiotic-resistant wound infections, Regeimbal and colleagues showed favorable efficacy of a phage cocktail against a particularly virulent clinical strain of *A. baumannii* (AB5075) in an insect-based bacterial virulence screening model (*Galleria mellonella*). This was translated into a mouse model of wound infection, showing reductions in animal weight loss, wound bioburden, and wound size resulting from the phage therapy. Importantly, the phage cocktail could only infect 10 of 92 screened clinical isolates of *A. baumannii*, illustrating the narrow spectrum of activity which poses a challenge to broad clinical use [58]. In spite of this issue, WRAIR investigators provided a phage cocktail (under FDA approval) which was used to successfully treat a critically ill human patient with a disseminated MDR *A. baumannii* infection who had failed conventional therapies [59]. Phage therapeutics for orthopedic device-related infections are currently being studied.

WRAIR investigators have also pursued a variety of novel small-molecule approaches to address the biofilm component of wound infections. Sambanthamoorthy and colleagues examined the antibiofilm activity of biologically produced surfactants from *Lactobacillus jensenii* and *L. rhamnosus* against MDR strains of *A. baumannii*, *E. coli*, and *S. aureus*. They found significant efficacy in both preventing new biofilms and dispersing established biofilms and observed minimal toxicity against cultured human lung epithelial cells at biofilm-effective concentrations [60]. The same investigators also performed in silico screening of a library of 15,000 compounds for inhibition of the bacterial enzyme diguanylate cyclase (which generates a signaling molecule), of which 250 were tested. Four compounds were found which inhibited biofilm formation by *P. aeruginosa* and *A. baumannii*, including one (LP 3134) which exhibited no cytotoxicity against cultured human keratinocytes [61]. Nine structural derivatives of this compound were subsequently explored for antibiofilm activity against *A. baumannii*, with seven of them effectively reducing biofilm formation on silicone catheters while exerting minimal toxicity to cultured human mammalian cells [62]. Finally, WRAIR investigators examined the activity of the cathelicidin peptide LL-37, and its metabolic fragments, against clinical isolates of MDR *A. baumannii* and their biofilms. While LL-37 and its KS-30 fragment were the most potent at reducing the biofilm, they also appeared to have limiting cytotoxicity. In contrast, the KR-20 fragment showed less efficient killing but was felt to be the most promising therapeutic candidate on the basis of its reduced cytotoxicity [63]. Perhaps these or other novel approaches provided by US military research laboratories can be successfully advanced to clinical development.

Conclusion

Recent military conflicts have included substantial challenges with wound infections, owing to MDRO phenotypes limiting antimicrobial treatment options, and likely involvement from bacterial biofilms contaminating devices implanted to stabilize severe extremity injuries. The US military medical research community responded to these challenges with a variety of in vitro and in vivo studies exploring the biology of these infections as well as potential novel mitigation strategies. The use of animal models of musculoskeletal infection, some of which include implanted devices contaminated with bacteria, has proven to be useful platforms for understanding optimal wound management strategies and screening compounds with promising in vitro antibiofilm activity. As an ideal antibiofilm agent has yet to be identified for clinical development, research in this field should continue in anticipation of wound infections being a significant clinical problem for casualties of future military conflicts.

Disclaimer The opinions or assertions contained herein are the private views of the author and not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

References

1. Jackman, R. P. (2013). Immunomodulation in transfused trauma patients. *Current Opinion in Anaesthesiology, 26*(2), 196–203.
2. Ake, J., et al. (2011). Gram-negative multidrug-resistant organism colonization in a US military healthcare facility in Iraq. *Infection Control and Hospital Epidemiology, 32*(6), 545–552.
3. Landrum, M. L., & Murray, C. K. (2008). Ventilator associated pneumonia in a military deployed setting: The impact of an aggressive infection control program. *The Journal of Trauma, 64*(2 Suppl), S123–S127; discussion S127–8.
4. Scott, P., et al. (2007). An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. *Clinical Infectious Diseases, 44*(12), 1577–1584.
5. Akers, K. S., et al. (2014). Biofilms and persistent wound infections in United States military trauma patients: A case-control analysis. *BMC Infectious Diseases, 14*, 190.
6. Owens, B. D., et al. (2007). Characterization of extremity wounds in Operation Iraqi Freedom and Operation Enduring Freedom. *Journal of Orthopaedic Trauma, 21*(4), 254–257.
7. Brown, K. V., Murray, C. K., & Clasper, J. C. (2010). Infectious complications of combat-related mangled extremity injuries in the British military. *The Journal of Trauma, 69*(Suppl 1), S109–S115.
8. Murray, C. K., et al. (2011). Prevention of infections associated with combat-related extremity injuries. *The Journal of Trauma, 71*(2 Suppl 2), S235–S257.
9. Masini, B. D., et al. (2011). Rehospitalization after combat injury. *The Journal of Trauma, 71*(1 Suppl), S98–S102.
10. Cross, J. D., et al. (2012). Return to duty after type III open tibia fracture. *Journal of Orthopaedic Trauma, 26*(1), 43–47.
11. Stinner, D. J., et al. (2010). Return to duty rate of amputee soldiers in the current conflicts in Afghanistan and Iraq. *The Journal of Trauma, 68*(6), 1476–1479.

12. Napierala, M. A., et al. (2014). Infection reduces return-to-duty rates for soldiers with Type III open tibia fractures. *Journal of Trauma and Acute Care Surgery, 77*(3 Suppl 2), S194–S197.
13. Huh, J., et al. (2011). Infectious complications and soft tissue injury contribute to late amputation after severe lower extremity trauma. *The Journal of Trauma, 71*(1 Suppl), S47–S51.
14. Tribble, D. R., et al. (2011). Infection-associated clinical outcomes in hospitalized medical evacuees after traumatic injury: Trauma Infectious Disease Outcome Study. *The Journal of Trauma, 71*(1 Suppl), S33–S42.
15. Weintrob, A. C., et al. (2018). Early infections complicating the care of combat casualties from Iraq and Afghanistan. *Surgical Infections, 19*(3), 286–297.
16. Johnson, E. N., et al. (2007). Infectious complications of open type III tibial fractures among combat casualties. *Clinical Infectious Diseases, 45*(4), 409–415.
17. Yun, H. C., Branstetter, J. G., & Murray, C. K. (2008). Osteomyelitis in military personnel wounded in Iraq and Afghanistan. *The Journal of Trauma, 64*(2 Suppl), S163–S168; discussion S168.
18. Eastridge, B. J., et al. (2009). Impact of joint theater trauma system initiatives on battlefield injury outcomes. *American Journal of Surgery, 198*(6), 852–857.
19. Tribble, D. R., et al. (2018). Osteomyelitis risk factors related to combat trauma open tibia fractures: A case-control analysis. *Journal of Orthopaedic Trauma, 32*, e344.
20. McDonald, J. R., et al. (2018). Infectious complications after deployment trauma: Following wounded United States military personnel into veterans affairs care. *Clinical Infectious Diseases, 67*, 1205.
21. Keeling, J. J., et al. (2008). Short-term outcomes of severe open wartime tibial fractures treated with ring external fixation. *The Journal of Bone and Joint Surgery. American Volume, 90*(12), 2643–2651.
22. Costerton, J. W., Montanaro, L., & Arciola, C. R. (2005). Biofilm in implant infections: Its production and regulation. *The International Journal of Artificial Organs, 28*(11), 1062–1068.
23. Costerton, J. W. (2005). Biofilm theory can guide the treatment of device-related orthopaedic infections. *Clinical Orthopaedics and Related Research*, (437), 7–11.
24. Forsberg, J. A., et al. (2008). Correlation of procalcitonin and cytokine expression with dehiscence of wartime extremity wounds. *The Journal of Bone and Joint Surgery. American Volume, 90*(3), 580–588.
25. Hawksworth, J. S., et al. (2009). Inflammatory biomarkers in combat wound healing. *Annals of Surgery, 250*(6), 1002–1007.
26. Utz, E. R., et al. (2010). Metalloproteinase expression is associated with traumatic wound failure. *The Journal of Surgical Research, 159*(2), 633–639.
27. Brown, T. S., et al. (2011). Inflammatory response is associated with critical colonization in combat wounds. *Surgical Infections, 12*(5), 351–357.
28. Evans, K. N., et al. (2012). Inflammatory cytokine and chemokine expression is associated with heterotopic ossification in high-energy penetrating war injuries. *Journal of Orthopaedic Trauma, 26*(11), e204–e213.
29. Sanchez, C. J., Jr., et al. (2013). Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infectious Diseases, 13*, 47.
30. Heitkamp, R. A., et al. (2018). Association of enterococcus spp. with severe combat extremity injury, intensive care, and polymicrobial wound infection. *Surgical Infections, 19*(1), 95–103.
31. Cardile, A. P., et al. (2014). Human plasma enhances the expression of Staphylococcal microbial surface components recognizing adhesive matrix molecules promoting biofilm formation and increases antimicrobial tolerance In Vitro. *BMC Research Notes, 7*, 457.
32. Akers, K. S., et al. (2015). Biofilm formation by clinical isolates and its relevance to clinical infections. *Advances in Experimental Medicine and Biology, 830*, 1–28.
33. Penn-Barwell, J. G., Murray, C. K., & Wenke, J. C. (2012). Comparison of the antimicrobial effect of chlorhexidine and saline for irrigating a contaminated open fracture model. *Journal of Orthopaedic Trauma, 26*(12), 728–732.

34. Svoboda, S. J., et al. (2006). Comparison of bulb syringe and pulsed lavage irrigation with use of a bioluminescent musculoskeletal wound model. *The Journal of Bone and Joint Surgery. American Volume, 88*(10), 2167–2174.
35. Owens, B. D., White, D. W., & Wenke, J. C. (2009). Comparison of irrigation solutions and devices in a contaminated musculoskeletal wound survival model. *The Journal of Bone and Joint Surgery. American Volume, 91*(1), 92–98.
36. Barsoumian, A., et al. (2013). In vitro toxicity and activity of Dakin's solution, mafenide acetate, and amphotericin B on filamentous fungi and human cells. *Journal of Orthopaedic Trauma, 27*(8), 428–436.
37. Homeyer, D. C., et al. (2015). In vitro activity of Melaleuca alternifolia (tea tree) oil on filamentous fungi and toxicity to human cells. *Medical Mycology, 53*(3), 285–294.
38. Yabes, J. M., et al. (2017). In Vitro activity of Manuka Honey and polyhexamethylene biguanide on filamentous fungi and toxicity to human cell lines. *Medical Mycology, 55*(3), 334–343.
39. Molinari, R. W., Khera, O. A., & Molinari, W. J., 3rd. (2012). Prophylactic intraoperative powdered vancomycin and postoperative deep spinal wound infection: 1,512 consecutive surgical cases over a 6-year period. *European Spine Journal, 21 Suppl 4*, S476–S482.
40. Pahys, J. M., et al. (2013). Methods to decrease postoperative infections following posterior cervical spine surgery. *The Journal of Bone and Joint Surgery. American Volume, 95*(6), 549–554.
41. Sweet, F. A., Roh, M., & Sliva, C. (2011). Intrawound application of vancomycin for prophylaxis in instrumented thoracolumbar fusions: Efficacy, drug levels, and patient outcomes. *Spine (Phila Pa 1976), 36*(24), 2084–2088.
42. Tennent, D. J., et al. (2016). Time-dependent effectiveness of locally applied vancomycin powder in a contaminated traumatic orthopaedic wound model. *Journal of Orthopaedic Trauma, 30*(10), 531–537.
43. Owens, B. D., & Wenke, J. C. (2007). Early wound irrigation improves the ability to remove bacteria. *The Journal of Bone and Joint Surgery. American Volume, 89*(8), 1723–1726.
44. Lack, W. D., et al. (2015). Type III open tibia fractures: Immediate antibiotic prophylaxis minimizes infection. *Journal of Orthopaedic Trauma, 29*(1), 1–6.
45. Zheng, Z., & Stewart, P. S. (2002). Penetration of rifampin through Staphylococcus epidermidis biofilms. *Antimicrobial Agents and Chemotherapy, 46*(3), 900–903.
46. Liu, C., et al. (2011). Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children: Executive summary. *Clinical Infectious Diseases, 52*(3), 285–292.
47. Shiels, S. M., et al. (2017). Determining potential of PMMA as a depot for rifampin to treat recalcitrant orthopaedic infections. *Injury, 48*(10), 2095–2100.
48. Shiels, S. M., Tennent, D. J., & Wenke, J. C. (2018). Topical rifampin powder for orthopaedic trauma part I: Rifampin powder reduces recalcitrant infection in a delayed treatment musculoskeletal trauma model. *Journal of Orthopaedic Research, 36*, 3136.
49. Rathbone, C. R., et al. (2011). Effect of various concentrations of antibiotics on osteogenic cell viability and activity. *Journal of Orthopaedic Research, 29*(7), 1070–1074.
50. Shiels, S. M., et al. (2018). Topical rifampin powder for orthopaedic trauma part II: Therapeutic levels of topical rifampin allows for spontaneous bone healing in both sterile and contaminated wounds. *Journal of Orthopaedic Research, 36*, 3142–3150.
51. Bhattacharjee, A., Nusca, T. D., & Hochbaum, A. I. (2016). Rhamnolipids mediate an interspecies biofilm dispersal signaling pathway. *ACS Chemical Biology, 11*(11), 3068–3076.
52. Kumar Shukla, S., & Rao, T. S. (2013). Dispersal of Bap-mediated Staphylococcus aureus biofilm by proteinase K. *Journal of Antibiotics (Tokyo), 66*(2), 55–60.
53. Roizman, D., et al. (2017). In vitro evaluation of biofilm dispersal as a therapeutic strategy to restore antimicrobial efficacy. *Antimicrobial Agents and Chemotherapy, 61*(10).
54. Cardile, A. P., et al. (2017). Activity of norspermidine on bacterial biofilms of multidrug-resistant clinical isolates associated with persistent extremity wound infections. *Advances in Experimental Medicine and Biology, 973*, 53–70.

55. Sanchez, C. J., Jr., et al. (2014). D-amino acids enhance the activity of antimicrobials against biofilms of clinical wound isolates of Staphylococcus aureus and Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy, 58*(8), 4353–4361.
56. Sanchez, C. J., Jr., et al. (2013). Effects of local delivery of D-amino acids from biofilm-dispersive scaffolds on infection in contaminated rat segmental defects. *Biomaterials, 34*(30), 7533–7543.
57. Be, N. A., et al. (2014). Microbial profiling of combat wound infection through detection microarray and next-generation sequencing. *Journal of Clinical Microbiology, 52*(7), 2583–2594.
58. Regeimbal, J. M., et al. (2016). Personalized therapeutic cocktail of wild environmental phages rescues mice from Acinetobacter baumannii wound infections. *Antimicrobial Agents and Chemotherapy, 60*(10), 5806–5816.
59. Schooley, R. T., et al. (2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. *Antimicrobial Agents and Chemotherapy, 61*(10).
60. Sambanthamoorthy, K., et al. (2014). Antimicrobial and antibiofilm potential of biosurfactants isolated from lactobacilli against multi-drug-resistant pathogens. *BMC Microbiology, 14*, 197.
61. Sambanthamoorthy, K., et al. (2014). Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development. *Biofouling, 30*(1), 17–28.
62. Sambanthamoorthy, K., et al. (2015). Modulating Acinetobacter baumannii biofilm development with molecules containing 3,4,5-trimethoxy-N,N′,N′-trimethylbenzohydrazide moiety. *Bioorganic & Medicinal Chemistry Letters, 25*(10), 2238–2242.
63. Feng, X., et al. (2013). The human antimicrobial peptide LL-37 and its fragments possess both antimicrobial and antibiofilm activities against multidrug-resistant Acinetobacter baumannii. *Peptides, 49*, 131–137.