ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2011, p. 3603–3608 Vol. 55, No. 7
0066-4804/11/$12.00 doi:10.1128/AAC.01606-10

In Vitro and *In Vivo* Activities of E-101 Solution against *Acinetobacter baumannii* Isolates from U.S. Military Personnel[▽]

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Received 18 November 2010/Returned for modification 27 December 2010/Accepted 15 April 2011

We evaluated the *in vitro* and *in vivo* activity of a novel topical myeloperoxidase-mediated antimicrobial, E-101 solution, against 5 multidrug-resistant *Acinetobacter baumannii* isolates recovered from wounded American soldiers. Time-kill studies demonstrated rapid bactericidal activity against all *A. baumannii* strains tested in the presence of 3% blood. The *in vitro* bactericidal activity of E-101 solution against *A. baumannii* strains was confirmed in a full-thickness excision rat model. Additional *in vivo* studies appear warranted.

Acinetobacter baumannii is an environmentally resilient organism found in hospital- and community-acquired infections. In recent years, multidrug-resistant (MDR) *A. baumannii* has emerged as an increasing threat, particularly for American soldiers wounded in Iraq and Afghanistan (6, 21). These isolates are highly resistant to currently available antimicrobials (12). Recent studies have shown E-101 solution to be a potent, broad-spectrum, and fast-acting bactericidal formulation effective against MDR organisms *in vitro* (8). The purpose of this study was to determine both the *in vitro* antibacterial activity and *in vivo* efficacy of E-101 solution against MDR isolates of *A. baumannii* associated with combat injuries.

E-101 solution is a topical agent developed as a supplement to the standard of care for the clinical management of traumatic wounds and the prevention of surgical site infections. E-101 solution is a defined, formulated, cell-free oxidant-generating coupled-enzyme system containing porcine myeloperoxidase (pMPO) and glucose oxidase (GO) from *Aspergillus niger*, glucose, sodium chloride, and specific stabilizing amino acids (Fig. 1). The substrate for this catalytic process is glucose. The enzymatic activity of GO directed to glucose results in production of hydrogen peroxide (H_2O_2) (serving as a substrate for catalysis of pMPO), oxidation of chloride to hypochlorous acid (HOCl), and production of H_2O_2 for a reaction with HOCl to produce singlet oxygen ($^1O_2$). The antimicrobial mechanism of action of E-101 solution is enhanced by the binding of pMPO to the surface of target microorganisms, thereby optimizing direct oxidative damage of microbes by focused production of HOCl, which reacts with an additional H_2O_2, producing $^1O_2$ (1, 2, 5, 10, 11, 17, 20). Singlet oxygen is a potent antimicrobial product of the myeloperoxidase system, but the short lifetime of $^1O_2$ (about 2 μs in water) restricts its kill radius to the width of the bacterial cell wall, thus precluding collateral damage to surrounding host cells and tissue (1, 2, 3, 13, 14, 18). Evidence of this lack of collateral damage is provided by data from comprehensive preclinical toxicity and experimental wound-healing models that demonstrate both a lack of injury to host cells and tissues and no delay in the normal wound healing processes. Given that E-101 solution is antimicrobial when applied topically and given that host cells do not experience collateral damage, E-101 solution is an excellent candidate for use as a topical agent to reduce the incidence of infection in traumatic wounds (e.g., those incurred in combat) and surgical wounds.

(This work was presented in part at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 12 to 15 September 2009).

Organisms. Five clinical isolates of MDR *A. baumannii* were provided by the U.S. Army Institute of Surgical Research (Fort Sam Houston, TX; CRADA W81XWH-05-0153). Four of the five strains (07-003, 07-004, 07-005, and 07-007) were isolated

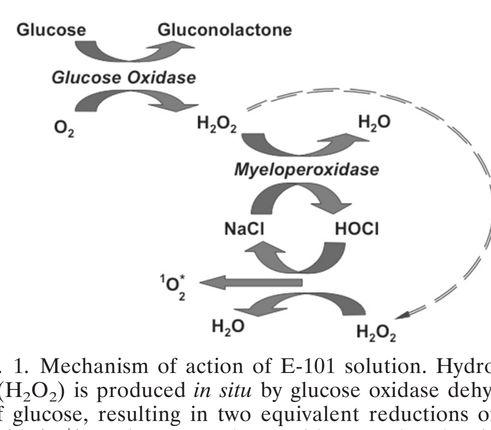

FIG. 1. Mechanism of action of E-101 solution. Hydrogen peroxide (H_2O_2) is produced *in situ* by glucose oxidase dehydrogenation of glucose, resulting in two equivalent reductions of oxygen. The acid (H^+) optimum myeloperoxidase-catalyzed oxidation of chloride ion (the Cl^- of NaCl) by H_2O_2 generates hypochlorous acid (HOCl). Once generated, HOCl (or its conjugate base OCl^-) participates in a diffusion-controlled reaction with a second H_2O_2 molecule to yield singlet oxygen ($^1O_2$). Singlet oxygen is a potent electrophilic oxygenating agent capable of reacting with a broad spectrum of electron-rich compounds. (Adapted from reference 8 with permission of the publisher.)

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▽ Published ahead of print on 25 April 2011.

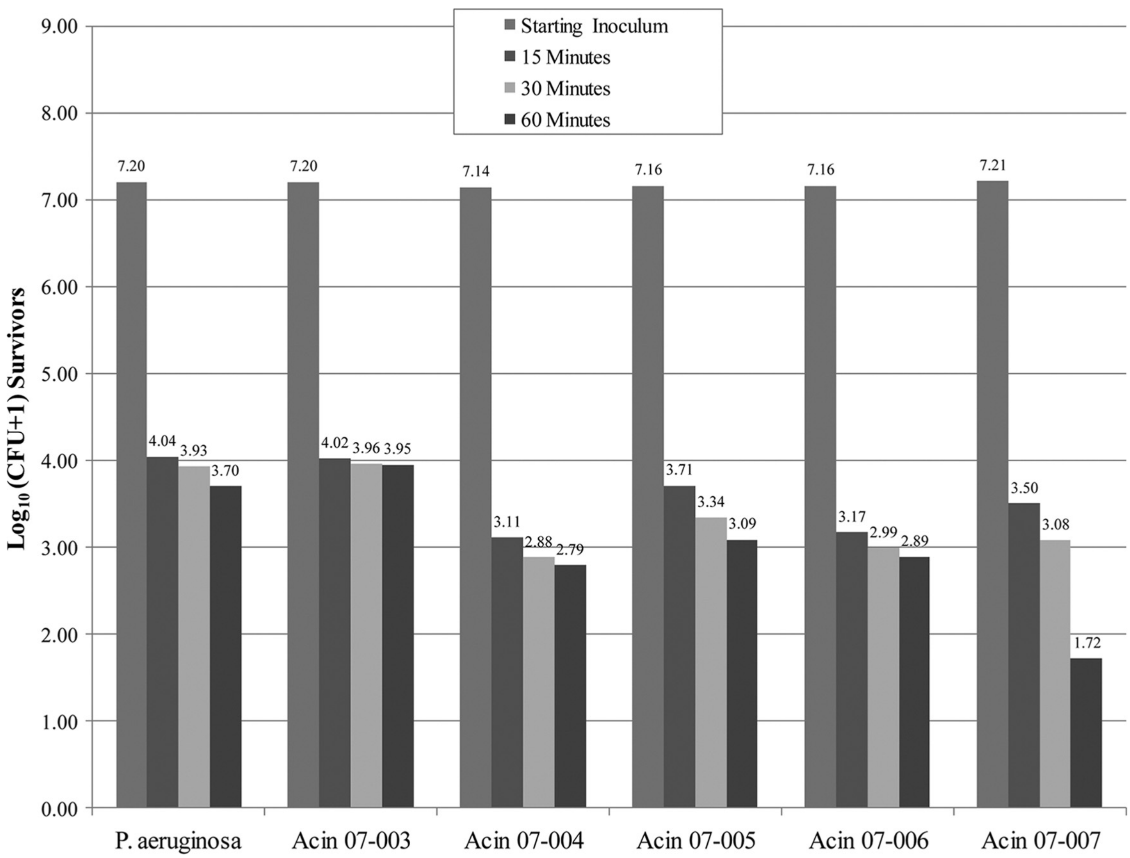

FIG. 2. *In vitro* activity of E-101 solution at 37.5 GU pMPO/ml against *A. baumannii* and *P. aeruginosa* in the presence of 3% rat blood. A greater than 3.0 $\log_{10}$ reduction of each *A. baumannii* strain population was achieved within 15 min.

from wounds, and a fifth strain (07-006) was isolated from blood. All MDR strains were resistant to more than 3 classes of antibiotics, including the carbapenems. Of 23 antibiotics tested, only colistin was active against all five strains. One reference clinical strain of *Pseudomonas aeruginosa* (R-463) with known susceptibility to E-101 solution was included for comparison.

E-101 solution. E-101 solution is composed of two aqueous solutions designated the enzyme solution and the substrate solution. The enzyme solution contains pMPO and GO derived from *Aspergillus niger* and proprietary amino acids in an aqueous formulation vehicle. The aqueous formulation consists of 150 mM sodium chloride and 0.02% (wt/vol) polysorbate 80–20 mM sodium phosphate buffer (pH 6.5). The substrate solution contains 300 mM glucose in the same aqueous formulation as the enzyme solution. The enzyme and substrate solutions are packaged in two separate vials and mixed together to activate the system. Once activated, E-101 solution should be used within 6 h of mixing when stored at 5 to 8°C. Short-term stability studies verify that E-101 solution maintains its microbicidal activity when prepared and maintained at room temperature for up to 90 min after mixing. E-101 solution formulations are based on the activity concentration of pMPO expressed as guaiacol units (GU) of pMPO per ml (GU pMPO/ml) according to an adapted assay by Chance and Maehly (7) performed to determine classical peroxidase activity. The classical peroxidase activity of myeloperoxidase (MPO) is proportional to its haloperoxidase activity. The relationship of GU activity to the weight of purified MPO is 375 GU/mg. The concentrations of GO and amino acids were directly proportional to that of pMPO (3:1 pMPO/GO ratio). The concentrations of the other components were held constant. The concentrations of E-101 solution tissue used in the *in vitro* studies were 37.50 and 150 GU pMPO/ml in the presence of 3% rat blood. The concentrations of E-101 solution used in the *in vivo* studies were 150 and 300 GU pMPO/ml, which represent proposed therapeutic doses for a phase 3 clinical study.

***In vitro* time-kill studies.** Time-kill studies were performed using a suspension-neutralization method (19) in the presence of 3% rat blood. Bacterial suspensions were prepared to achieve late log to early stationary-phase growth. The *in vitro* assays were conducted using glass vials. The test volume was 1.0 ml, which included activated E-101 solution, the bacterial suspension at a final target concentration of approximately 10^7 CFU/ml, and 30 μl of rat blood. Reaction vials were incubated at room temperature, and the enzyme activity was stopped by the addition of 100 μl of a sterile 1% catalase solution at 5, 15, 30, or 60 min. Samples were collected from the reaction vials, and quantitative culture experiments were performed in duplicate. The average numbers of $\log_{10}$ CFU + 1 survivors at each E-101 solution concentration were determined versus time.

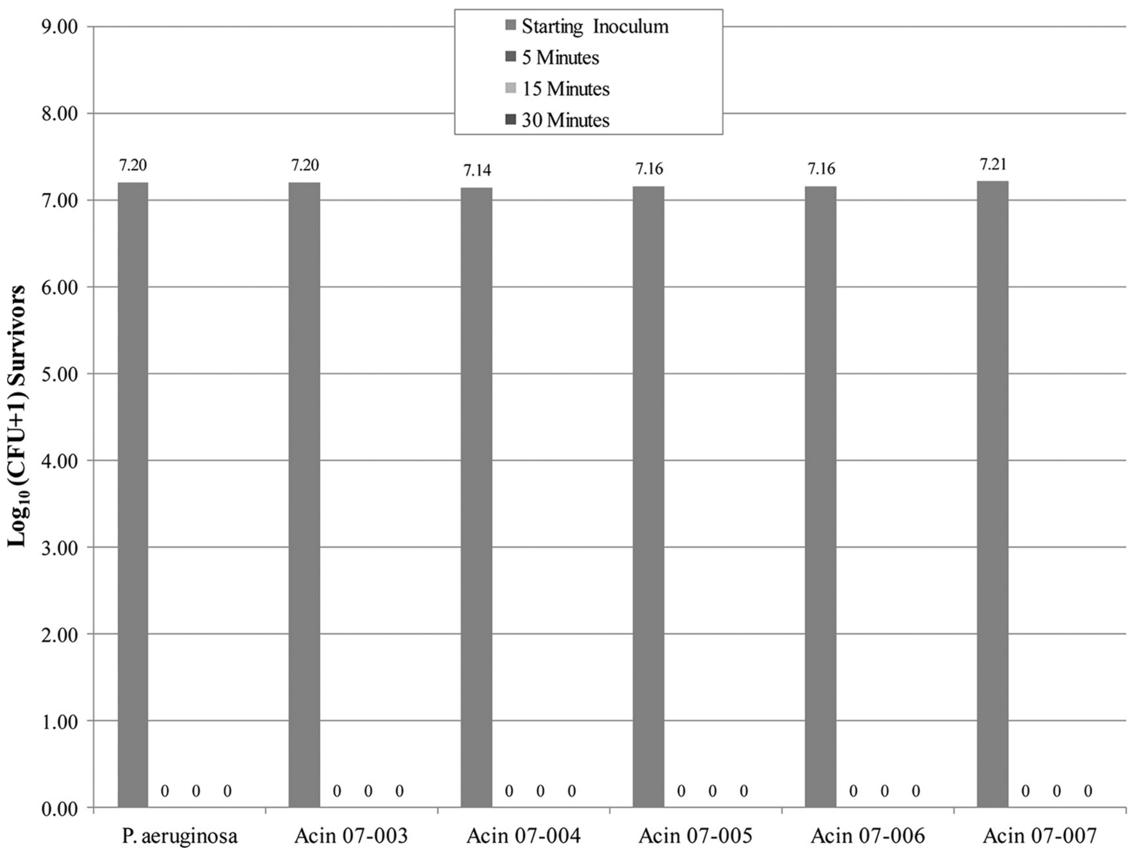

FIG. 3. *In vitro* activity of E-101 solution at 150 GU pMPO/ml against *A. baumannii* and *P. aeruginosa* in the presence of 3% rat blood. No detectable *A. baumannii* and *P. aeruginosa* survivors were observed within 5 min.

***In vivo* wound model.** Experimental wounds were produced by a modification of the method reported by Saymen et al. (15). Two full-thickness excision wound sites were prepared on the backs of anesthetized adult male Sprague-Dawley rats by exposing a 1-cm by 1.5-cm area of fascia. Three rats with 2 wounds each were used for each treatment group. An open 2.5-cm-diameter polystyrene cylinder was glued to the skin around each excised site with Quick Tite (Loctite Corp.) cement as described by Breuing et al. (4). Each cylinder formed a liquid-tight test chamber, the base of which was formed by the exposed fascia. The exposed fascia was inoculated by depositing 200 μl (containing 10^7 CFU) of each bacterial suspension. The inoculum was allowed to remain on the fascia for 15 min before application of activated E-101 solution. A volume of 800 μl of E-101 solution was introduced into the test chamber, resulting in a total volume of 1.0 ml per test site. Recovery control sites were treated with 800 μl of 0.9% sterile saline solution. After 15-and 30-min exposure times with E-101 solution, 100 μl of a 10% solution of catalase was added to each test chamber to neutralize any further enzymatic activity of E-101 solution, thereby inhibiting further microbicidal activity by this mixture. The liquid in the cylinder was then recovered, and the underlying fascia was aseptically excised, weighed, and homogenized. Quantitative cultures of liquid sample and tissue homogenate were prepared by plating serial 10-fold dilutions of each sample, and colonies were counted to determine organism survival levels. Undiluted samples (1.0 ml) for both *in vitro* and *in vivo* studies were also plated, with the lowest level of detection being 1.0 CFU/ml.

Statistics. The performance of the E-101 solution treatment was calculated as the sum of CFUs from the recovered liquid and tissue homogenate for each wound and is reported as the numbers of mean survivors based on 3 rats per concentration for each time point, where the value used for each rat is based on the mean value determined for two sites. The mean $\log_{10}$ reduction value ($\log_{10}$ inoculum $-$ $\log_{10}$ CFU + 1 survivors) was used for comparison. A *t* test was used to assess the change from the starting inoculum within each treatment group at each time point. The differences between the effects of two concentrations of E-101 solution (150 GU pMPO/ml and 300 GU pMPO/ml) at each time point were also tested for statistical significance by the *t* test.

E-101 solution exhibited concentration- and time-dependent *in vitro* activity in the presence of 3% rat blood. At 37.5 GU pMPO/ml, a reduction of more than 3 $\log_{10}$ was achieved within 15 min for each *A. baumannii* strain (Fig. 2). At 150 GU pMPO/ml, no detectable *A. baumannii* survivors were observed at 5 min (Fig. 3). The *in vitro* activity of E-101 against *A. baumannii* strains was comparable to the activity seen against *P. aeruginosa.*

The *in vivo* antimicrobial activity of E-101 solution against MDR *A. baumannii* strains was demonstrated in the rat full-thickness excision model. After 15 and 30 min of treatment with 150 GU pMPO/ml and 300 GU pMPO/ml, the mean

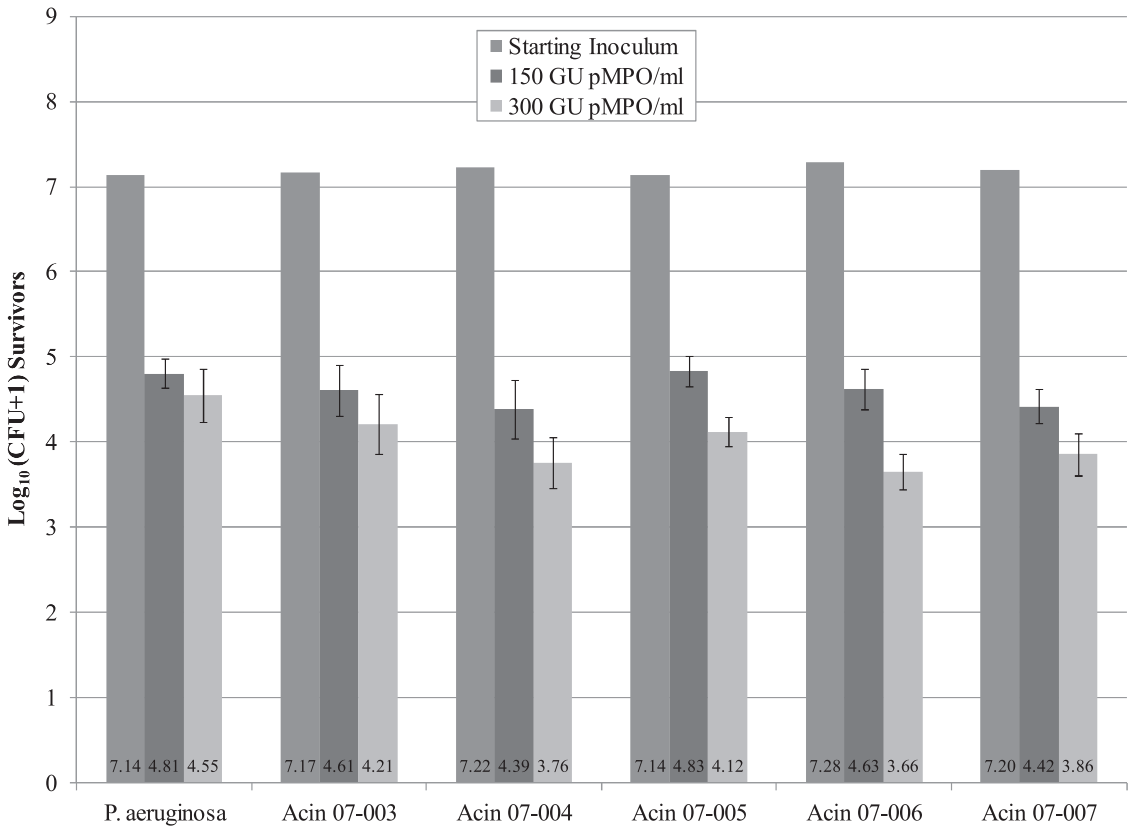

FIG. 4. *In vivo* activity of E-101 solution against *A. baumannii and P. aeruginosa* 15 min after administration of a single dose at 150 GU pMPO/ml and 300 GU pMPO/ml in the rat full-thickness excision model. The data represent the means and standard deviations based on 3 rats per treatment group, where the value used for each rat represents the average of data for 2 wound sites. Standard deviation bars are not included for the starting inoculum, since there was no variation across the members of the rat groups. The mean number of CFU was significantly ($P < 0.01$) reduced from baseline (starting inoculum) for each organism. A 2.5 and 3.0 $\log_{10}$ reduction in CFU was observed within 15 min at 150 GU pMPO/ml and 300 GU pMPO/ml, respectively. Additionally, the antimicrobial activity of E-101 solution at 300 GU/pMPO/ml significantly ($P <$ 0.0001) reduced survivor numbers compared to 150 GU pMPO/ml when results for all five organisms were pooled.

number of CFUs was significantly reduced from the baseline (starting inoculum) for each organism. The highest P value among all the t tests for each of the five *A. baumannii* strains was <0.0105. Reductions in CFU numbers of approximately 2.5 and 3.0 $\log_{10}$ were observed within 15 min of administration of 150 GU pMPO/ml and 300 GU pMPO/ml, respectively (Fig. 4). A greater than a 3.0 $\log_{10}$ reduction in CFU was observed within 30 min at 300 GU pMPO/ml (Fig. 5). The antimicrobial activity of E-101 solution at 300 GU pMPO/ml significantly reduced survivor numbers compared to 150 GU pMPO/ml at the 15- and 30-min time points when results for all five organisms were pooled ($P < 0.0001$). Although there appeared to be a concentration- and time-dependent kill effect *in vivo*, the extent of killing reached a plateau after 30 min with both formulations after treatment with a single dose. The one blood isolate of *A. baumannii* (07-006) appeared more susceptible to E-101 solution than those obtained originally from combat wounds. MPO shows selective microbial binding and, as such, selective microbicidal action. In that regard, pMPO showed strong binding and microbicidal action against all Gram-negative bacteria tested (1, 2).

E-101 solution remains functionally microbicidal in the presence of moderate concentrations of blood contamination as observed in dose-response studies with *Staphylococcus aureus* in the presence of 24% blood. As previously demonstrated, the MPO microbicidal system of E-101 solution is more potent than either H_2O_2 or OCl^- in the presence of blood erythrocytes (1). However, the formulation is neutralized by undiluted blood. The neutralizing effect of blood on MPO results from blood's alkaline pH of 7.4 and the presence of erythrocytes rich in catalase as well as plasma rich in ceruloplasmin. MPO haloperoxidase activity is optimal in an acid pH, is in competition with catalase for available H_2O_2, and is inhibited by ceruloplasmin. Ceruloplasmin is the major copper-containing protein in serum and has previously been reported to selectively bind to and inactivate MPO *in vivo* (16). Hence, the primary clinical development focus for the use of E-101 solution is direct topical application into traumatic wounds (e.g., those incurred in combat) and surgical wounds.

E-101 solution is a cell-free system that generates reactive oxidants such as HOCl (or its conjugate base OCl^-) and $^1O_2$ when MPO, chloride, and a source of H_2O_2 are present (Fig. 1). It is formulated to mimic the intrinsic *in situ* functions of the phagolysosome (9). It performs as a stable microbicide when applied directly into a wound or surgical incision site. The microbicidal combustive action of E-101 solution against target

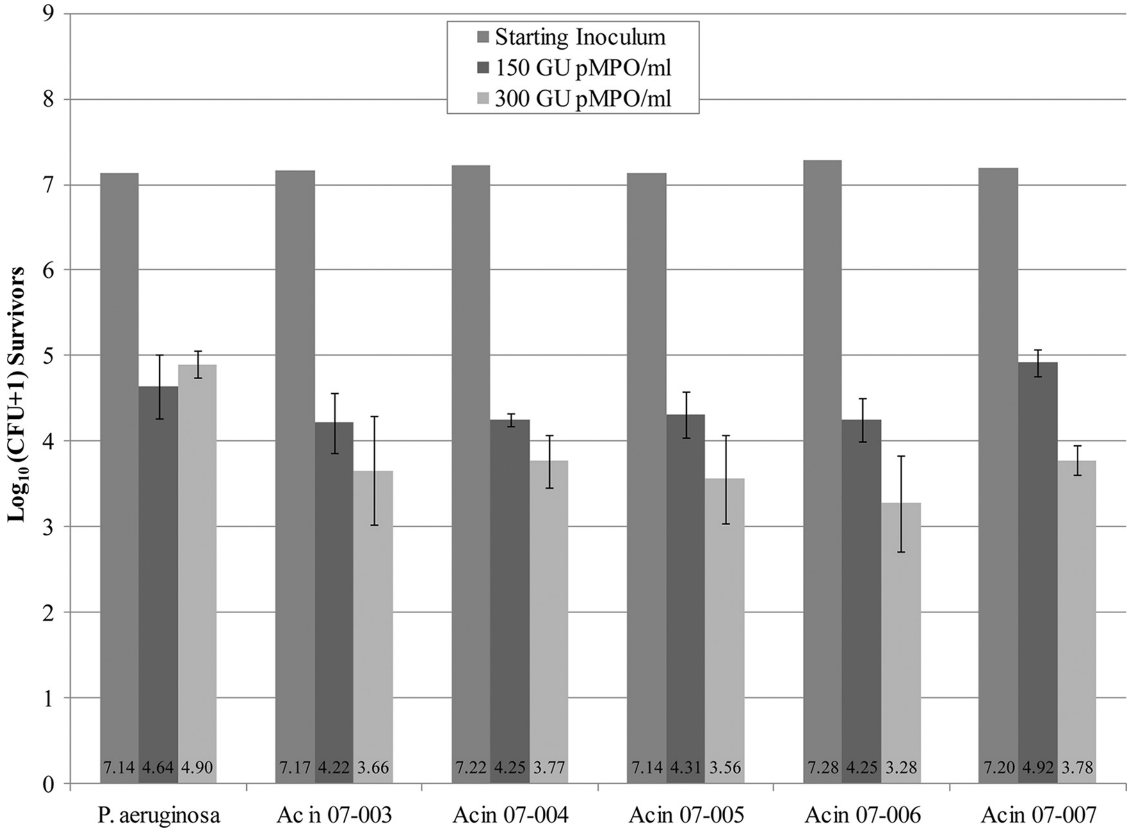

FIG. 5. *In vivo* activity of E-101 solution against *A. baumannii and P. aeruginosa* 30 min after administration of a single dose at 150 GU pMPO/ml and 300 GU pMPO/ml in the rat full-thickness excision model. The data represent the means and standard deviations based on 3 rats per treatment group, where the value used for each rat represents the average of data for 2 wound sites. The mean number of CFU was significantly ($P < 0.0105$) reduced from baseline (starting inoculum) for each organism. A greater than a 3.0 $\log_{10}$ reduction in CFU of *A. baumannii* was observed within 30 min. Additionally, the antimicrobial activity of E-101 solution at 300 GU pMPO/ml significantly ($P < 0.0001$) reduced survivor numbers compared to 150 GU pMPO/ml when results for all five organisms were pooled.

microorganisms is directed to a variety of molecular and enzymatic sites that are essential for metabolism or for the integrity of the microorganism (20). As previously demonstrated, MPO-H_2O_2 microbicidal activity is several orders of magnitude more potent than that of H_2O_2 alone and this activity is more resistant to erythrocyte inhibition than the activity of either H_2O_2 or OCl^- (1). The rapid rate of killing induced by E-101 solution is consistent with its combustive oxygenation mechanism of action. In addition to catalase, which competitively destroys H_2O_2., erythrocyte contains molecular substrates that competitively react with available $^1O_2$ and OCl^-, thus inhibiting the action of E-101 solution. Increasing the dosage of E-101 solution partially overcomes blood interference.

The sources of *A. baumannii* infections in patients with traumatic injuries are most likely environmental contamination of wounds in the field or nosocomial spread during treatment at medical facilities (6). In the full-thickness excision model, E-101 solution demonstrated a rapid decrease in bacterial inoculum numbers after a single dose, which should increase the effectiveness of standard wound care. The distinct advantages of using E-101 solution in the prevention of wound infections are the demonstrated rapid antimicrobial activity and its broad spectrum of activity (8). The need for new agents to treat MDR pathogens and the efficacy of E-101 solution against MDR *A. baumannii* support further studies to assess its utility for wound decontamination and for prevention of infection in traumatic and surgical wounds. Future studies are planned to include efficacy testing in the presence of increasing concentrations of blood, selection of a deep-wound model with extended-duration evaluation times, and multiple applications of E-101 solution in the animal models.

We thank Robert C. Allen for his useful insights and helpful discussions. Statistical analysis and preparation of figures were provided by Veristat, Inc. Holliston, MA.

This work was supported by Exoxemis, Inc., Little Rock, AR.

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