Chapter 25

Skin and Soft Tissue Models for *Acinetobacter baumannii* Infection

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Abstract

Multidrug-resistant *A. baumannii* are important Gram-negative pathogens causing persistent wound infections in both wounded and burned victims, which often result in secondary complications such as delayed wound healing, skin graft failure, and sometimes more serious outcomes such as sepsis and amputation. The choice of antibiotics to remediate these *A. baumannii* infections is becoming limited; and therefore, there has been a renewed interest in the research and development of new antibacterials targeting this pathogen. However, the evaluation of safety and efficacy is made more difficult by the lack of well-established in vivo models. This chapter describes established rodent and large animal models that have been used to investigate and develop treatments for *A. baumannii* skin and soft tissue infections.

Key words ESKAPE pathogens, Drug-resistant bacteria, Graft failure, Animal models, SSTI, Wound healing, Mice, Pigs, Murine, Porcine

1 Introduction

Acinetobacter baumannii is a Gram-negative bacterial pathogen that is one of the key nosocomial bacterial species responsible for antibiotic-resistant infections that is a burden to healthcare facilities worldwide [1–3]. *A. baumannii* can persist for months in a desiccated state and, therefore, can be readily transmitted in the hospital environment as well as in nursing homes [4, 5]. This is further complicated by the fact that during the last few years, strains of *A. baumannii* that are resistant to the majority of currently available antibiotics have emerged and have been defined as multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) [3, 6]. MDR *A. baumannii* has been increasingly linked to skin and soft tissue infections (SSTI) [7] and is an important pathogen causing persistent wound infections in burn victims, leading to loss of skin grafts and delayed wound healing [8–10]. The US military healthcare system has also recorded a

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significant increase in the incidence of MDR *A. baumannii* in combat-related wound infections in the last 10–15 years [11, 12]. The majority of those infections occurred during the conflict in Iraq and coincided with polytraumatic injuries, where osteomyelitis, amputation, and amputation revisions became the norm in addition to SSTI [13]. Further, there have been increases in the incidence of neonate sepsis caused by *A. baumannii* [14, 15], some of which could be linked to a wound infection at the cesarean section site [15, 16].

Unfortunately, many cases have left physicians with only colistin, a drug of last resort, to remediate *A. baumannii* infections; and therefore, the lack of treatment options has driven the consideration of narrow-spectrum approaches and the development of new drugs targeting this pathogen [17]. Eliminating such infections requires a deeper understanding of the factors that enable the pathogen to persist as well as the ability to design and create key tests for new antimicrobials targeted specifically against *A. baumannii*. However, the research to develop these new drugs could be limited by a lack of well-established in vivo models. This review describes the rodent and large animal *A. baumannii* infection SSTI models available along with the development of the first preclinical, mono-infection models for *A. baumannii* infection developed in our lab (*see* **Note 1**). These models could become standardized approaches for translational research, but there are, of course, limitations to these approaches as well.

One well-accepted limitation of rodent models, with regard to wound infection, is the dissimilarity in physiology and anatomy with the human skin. Rodents have an increased amount of hair coupled with thinner dermal and epidermal layers. Rodents also lack a layer of fat cells underneath the dermis, which is found in humans. Furthermore, rodents are generally recognized to heal through a combination of both contraction and reepithelialization [18]. In contrast, humans heal by reepithelialization only [18]. From an anatomic and physiologic standpoint, porcine skin bears more similarities to humans in terms of thickness, cellularity, elasticity, healing times, hair follicle distribution, vessel size, and orientation [19, 20]. The porcine mono-infection model is, therefore, highly relevant with regard to skin infection and wound healing and will be an increasingly important tool as several commercial and academic scientists are working on small-molecule and antibiotic alternatives that have specific activity against *A. baumannii* to include systemic and topical applications [21].

Another challenge with *A. baumannii* infection models is the choice of strain(s) for the model system. Numerous groups have developed wound models (Table 1) using different clinical isolates or ATCC laboratory strains; however, virulence is a key variable and can differ greatly between strains [22], which, in turn, can confound antibacterial evaluation. We found it was important to use a more

Table 1
***A. baumannii* wound models of infection**

SPECIES	TYPE OF WOUND	BACTERIA STRAIN	PURPOSE	REF
Mouse - Female BALB/c	**Partial thickness skin abrasion** - Scraped with no. 15 scalpel blade resulting in a wound measuring approx. 1.2 cm × 1.2 cm.	Bioluminescent multidrug resistant clinical isolate *A. baumannii* strain. No specific strain mentioned.	Investigate the use of ultraviolet C light for prevention of infection	[28]
Mouse - Female BALB/c (6-8 wks)	**Full thickness excisional** – 5 mm diameter.	AB0057	Test the capacity of a nitric oxide-releasing nanoparticle to treat infected wounds.	[29]
Mouse - Female BALB/c (6-8 wks)	**Full thickness excisional** – 6 mm diameter. (described in detail in this review)	AB5075	Test antibiotics in a mon-infection model.	[23]
Mouse – athymic nude mice	**Burn**– 12% full-thickness dorsal scald burn injury induced by immersion of the dorsal skin in a 90 °C water bath for 10 seconds	No specific strain mentioned.	Test the efficacy of a genetically engineered human tissue expressing human cathelicidin host defense proteins.	[30]
Mouse - Female BALB/c (6-7 wks)	**Burn -** brass block at 95°C at dorsal surface for 7 seconds, resulting in full-thickness, third-degree burns measuring approximately 1.2 cm× 1.2 cm.	Bioluminescent multidrug resistant clinical isolate *A. baumannii* strain. No specific strain mentioned.	Test the utility of antimicrobial blue light therapy	[31]
Mouse - Female CD-1	**Burn** - pressing a 1 g hot metal weight (diameter 11 mm, temperature 90'C) to the shaved dorsal skin for 2 min.	ATCC BAA-1805	Test the efficacy of a designer antibacterial peptide with that of imipenem and colistin	[32]

(continued)

Table 1
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Mouse – Female C57BL/6, 4-month-old	**Burn** - dorsal skin surface contact for 10 seconds with brass blocks at 95°C, resulting in a nonlethal 1 cm^2, full-thickness burn.	ATCC BAA 747	Test a wound disinfection method using high voltage, short pulsed electric fields	[33]
Mouse - Female BALB/c	**Burn** - applying a brass block at 95°C to the dorsal surface for 7 secs, resulting in full-thickness, third-degree burns measuring approx. 1 cm × 1.5 cm	Bioluminescent multidrug resistant clinical isolate *A. baumannii* strain	To investigate the use of ultraviolet C light for prevention of infection	[28]
Mouse – Male C57BL/6J (7–8 wks)	**Burn sepsis -** 12 % dorsal scald burn injury induced by submerging the back in hot water at 85°C for 8 seconds, resulting in a full-thickness burn.	No specific strain mentioned.	Test the efficacy of a novel antibacterial therapeutic technology using bacterial conjugation (carried within *Escherichia coli*)	[34]
Mouse – BALB/c	**Systemic infection**	ATCC 17978, HUMC1, HUMC4, HUMC5, HUMC6, HUMC12	Identify a recombinant vaccine immunogen that protected mice against lethal infection, and also induced protective antibodies when administered as passive immunization	[35]
Rat - Male obese Zucker	**Full thickness excisional -** surface area 4.6 cm^2	ATCC 19606	Test the topical delivery of nitric oxide (NO) through a wound dressing for its potential to reduce wound infections	[36]
Rat	**Full thickness excisional –** 1.5cm X 1.5 cm	T-10 and G7	Test the effectiveness of phage administration as an antibacterial remedy	[37]

(continued)

Table 1
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Rat - Sprague-Dawley (250–300 g)	**Burn** –skin contact with water at 100'C for 10 s, resulting in a full thickness burn	ATCC 19606	Compare the efficacy of different topical antibacterial agents	[38]
Pig – Female Yorkshire (25-35 kg)	**Burn -** electrically heated burn device with controlled pressure delivery at 150°C heated stylus for 50 seconds, resulting in 2"x2" inch full thickness burns.	ATCC 19606	To study polymicrobial wound biofilm and host interaction	[39]
Pig – Female Yorkshire (25-35 kg)	**Full thickness excisional** – 12 mm diameter (described in detail in this review).	AB5075	Mono-species to evaluate topical polymyxin B	[40]

Shaded rows are the models discussed in further detail in this review

virulent isolate and the same strain between all models for consistency when evaluating novel antibacterials. AB5075, an ST2 strain, better represents the isolates found in the clinic with increased antibiotic resistance and negative outcomes being observed in patients [22] (*see* **Note 2**). In this chapter, we will present our two excisional wound models in both murine and porcine species. These models rely on the increased and consistent virulence of AB5075 across animal systems and the use of cyclophosphamide to dampen the innate immune response [22] (*see* **Note 3**).

2 Materials

2.1 Murine Wound Infection Model

1. Female BALB/c mice (6–10 weeks of age) (14–20 g weight) (*see* **Note 4**).
2. 150 and 100 mg/kg cyclophosphamide in 0.9% sodium chloride injection solution (*see* **Note 5**) (final concentration—100 mg/mL).

3. 130 mg/kg ketamine.
4. 10 mg/kg xylazine.
5. 28 gauge syringe.
6. 0.05 mg/kg buprenorphine.
7. Electric razor.
8. Iodine solution.
9. Ethanol rinse solution.
10. 6.0 mm disposable skin biopsy punch.
11. 2.0×10^6 CFU/mL AB5075 *A. baumannii* cells in PBS suspension.
12. Lennox Luria-Bertani (LB) media.
13. Tegaderm roll, 3M Health Care.
14. Vetbond tissue adhesive.
15. Sterile water and food—dry rodent chow supplemented with DietGel Recovery (ClearH_2O, Portland, ME) (*see* **Note 6**).

2.2 Porcine Wound Infection Model

1. Female Yorkshire pigs (30–35 kg).
2. 12–20 mg/kg ketamine.
3. 2–4.4 mg/kg xylazine.
4. Isoflurane gas.
5. Sterile water and food—Laboratory Porcine Diet Grower 5084 (Purina LabDiet®, St. Louis, MO).
6. 25 mg/kg cyclophosphamide IV in 0.9% sodium chloride injection solution (final concentration—100 mg/mL).
7. Electric razor.
8. Chlorhexidine rinse.
9. 12 and 4 mm disposable skin biopsy punch.
10. Gauze.
11. 1.0×10^6 CFU/mL AB5075 *A. baumannii* cells in PBS suspension.
12. Lennox Luria-Bertani (LB) media.
13. Tegaderm roll, 3M Health Care.
14. Vetbond tissue adhesive.
15. 75 mcg/h fentanyl patch.
16. Bandaging tape (VetrapTM).

3 Methods

3.1 Murine Monospecies Wound Infection Model

Purpose: A *murine full-thickness, excisional wound model* was developed in which a diminutive inoculum of a clinically relevant, multidrug-resistant *A. baumannii* isolate was inoculated that could proliferate, form biofilms, and be effectively treated with antibiotics [23] (Figs. 1 and 2).

1. Female BALB/c mice were purchased from Charles River Laboratories (Frederick, MD). The mice used in these experiments were 6–10 weeks of age and weighed 14–20 g.
2. Cyclophosphamide (Baxter, Deerfield, IL) was dissolved in 0.9% sodium chloride injection solution (Hospira Inc., Lake Forest, IL) to obtain a final concentration of 100 mg/mL. Mice received 150 mg/kg (*see* **Note 5**) of body weight and 100 mg/kg cyclophosphamide via intraperitoneal (i.p.) injections, before wounding and infection, on days −4 and −1, respectively.
3. On day 0, the day of wounding and inoculation, mice were anesthetized with an injection of a mixed ketamine (130 mg/kg) and xylazine (10 mg/kg) solution i.p. with a 28-gauge syringe. Buprenorphine (0.05 mg/kg) was administered separately via intramuscular (i.m.) injection for pain management.
4. Hair was clipped with an electric razor from the cervical to mid-lumbar dorsum, and the skin was scrubbed with iodine solution followed by an ethanol rinse.
5. A 6.0 mm disposable skin biopsy punch was used to create a full-thickness skin defect overlying the thoracic spinal column and the adjacent musculature. The circular skin to be removed is lifted with forceps, and any subepithelial connective tissue is incised with iris scissors (*see* **Note 7**).
6. Aliquots of 50 μL containing 5.0×10^4 AB5075 cells in a PBS suspension were pipetted into the wound and allowed to absorb for 3 min.
7. A circular cutout (30 mm in diameter) of transparent dressing (Tegaderm roll; 3M Health Care, St. Paul, MN) was placed over the wound and secured with tissue adhesive (Vetbond; 3M Animal Care, St. Paul, MN) (*see* **Note 8**).
8. Beginning at 4 h post-inoculation, mice are treated with any antibiotic treatments once daily for a 6-day treatment period.
9. All mice received sterile food and water ad libitum, and dry rodent chow was supplemented with DietGel Recovery (ClearH_2O, Portland, ME) during 48 h following wounding.
10. On day 7, the transparent dressing was removed, the treatment was discontinued, and the wound was monitored for closure through day 25.

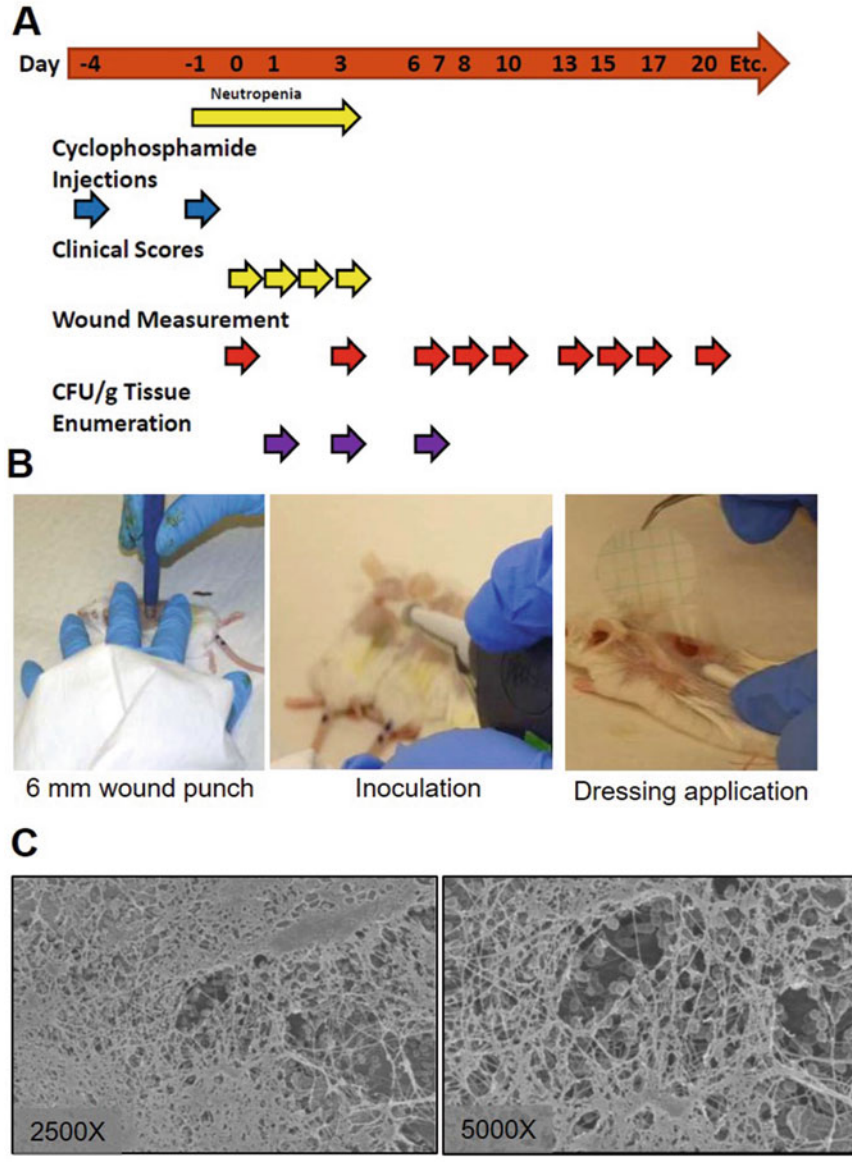

Fig. 1 Murine excisional *A. baumannii* wound model. (**a**, **b**) The murine dorsal wound puncture model uses 6–10-week-old female BALB/c mice that are treated with cyclophosphamide at 4 days prior to infection with (150 mg/kg) cyclophosphamide and again 1 day prior to infection (100 mg/kg). On the day of surgery, mice are weighed and anesthetized with ketamine/xylazine. Backs are shaved and sterilized with a povidone-iodine scrub followed by washing with 70% isopropanol. A full-thickness 6 mm diameter punch is taken from the back of the mouse. Once the wound is created, the mouse is administered a localized dose of buprenorphine and then inoculated with 5.0×10^4 cells of AB5075. To enclose the wound, a circular piece of Tegaderm™ dressing is applied with supplemental glue over the puncture with Vetbond, and the mice are allowed to recover on a heating pad. (**c**) Biofilm formation at 24 h post-infection analyzed using scanning electron microscope (left) 2500× zoom and (right) 5000× zoom

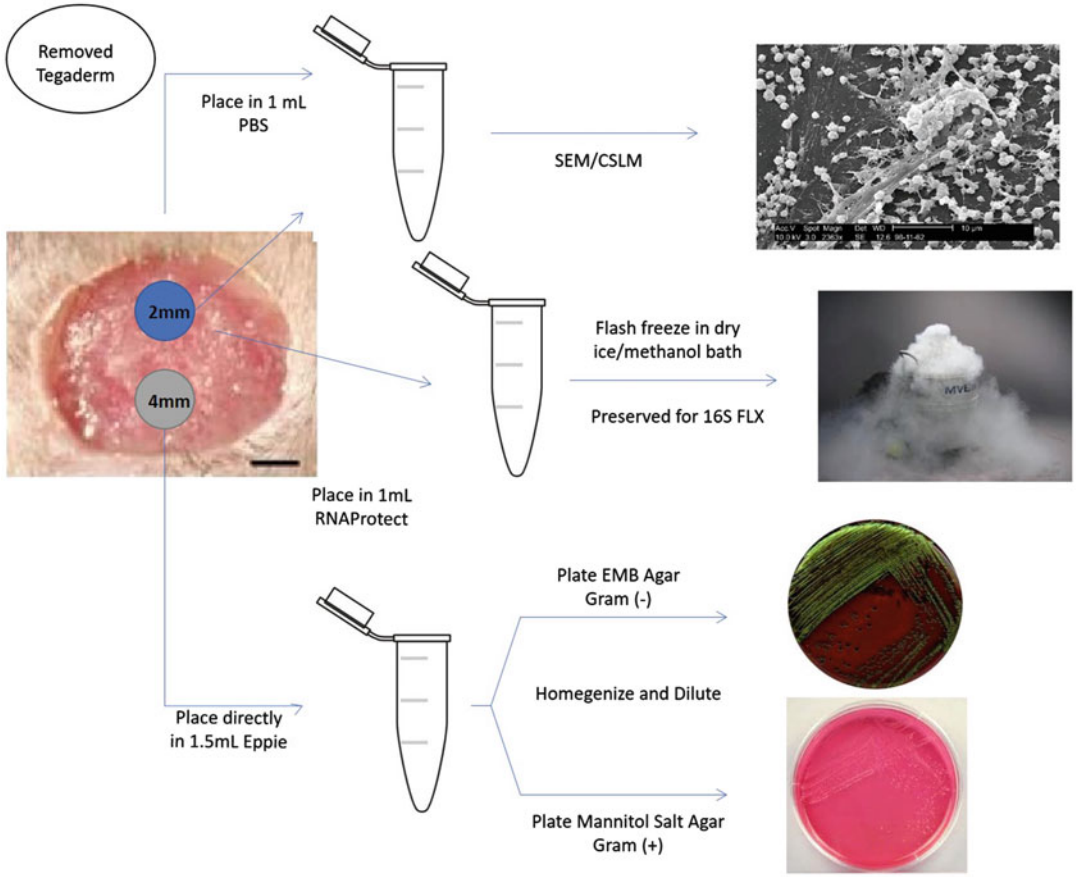

Fig. 2 Collection of biopsies from the murine wound

3.2 Porcine Mono-species Wound Infection Model

Purpose: An *excisional, mono-species infected porcine wound model* was developed, in which a diminutive inoculum (5.0×10^4 CFU) of a clinically relevant MDR *A. baumannii* isolate could proliferate, form biofilms, and be effectively treated with antibiotics (Figs. 3 and 4). This model can therefore simulate a skin and soft tissue wound infection preclinical model and can be used to assess novel antimicrobial compounds targeted specifically to *A. baumannii*.

1. Female Yorkshire pigs weighing 30–35 kg (Animal Biotech Industries, Doylestown, PA) were used. All pigs received measured amounts of Laboratory Porcine Diet Grower 5084 (Purina LabDiet®, St. Louis, MO) and water ad libitum.
2. Beginning on day −4 and at all subsequent time points (days 0, 1, 3, 7, and 10), pigs were anesthetized with ketamine 12–20 mg/kg and xylazine 2.0–4.4 mg/kg intramuscular (i.m.) injection, followed by tracheal intubation and anesthetic maintenance on 2.0–4.0% isoflurane gas.

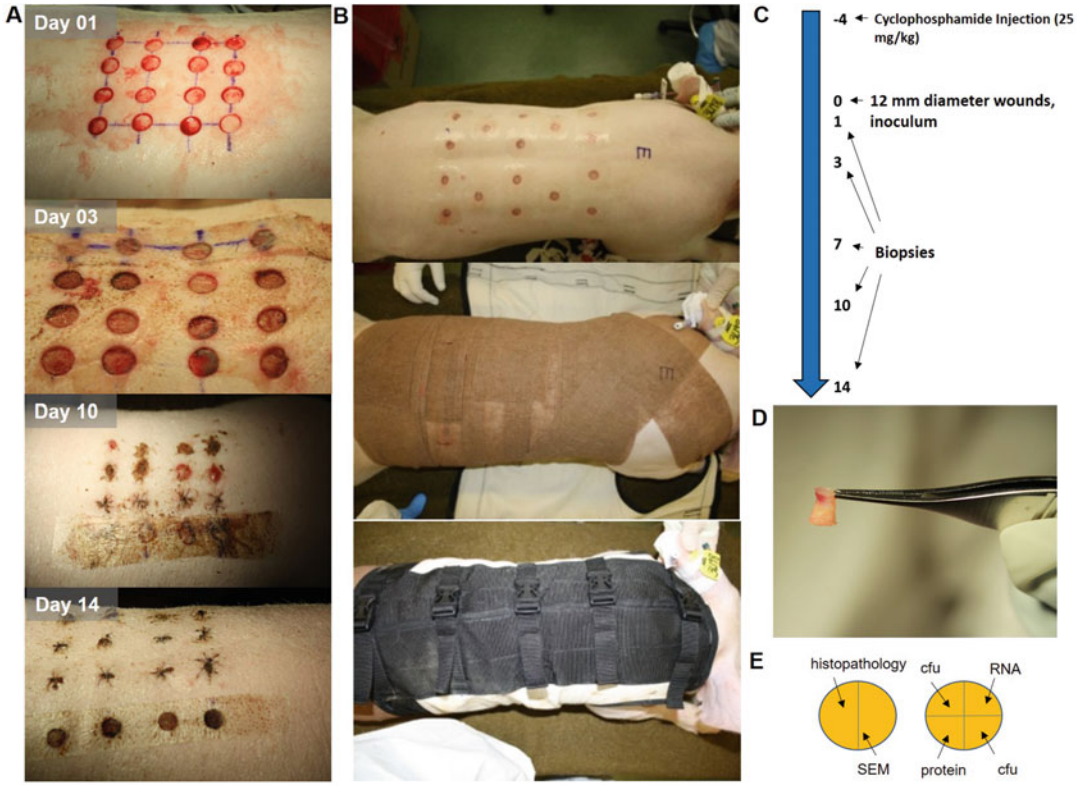

Fig. 3 Porcine excisional *A. baumannii* wound model. (**a**) The porcine dorsal wound puncture model uses Yorkshire pigs (female, 30–35 kg). The pigs are given a single 25 mg/kg intravenous cyclophosphamide injection at day −4. On day 0, anesthesia is induced, and 16 full-thickness dorsal skin wounds in a 4 × 4 grid formation are created using a 12 mm diameter biopsy punch. Each wound bed is inoculated with 5.0×10^4 CFU AB5075 and then covered with Tegaderm™ dressing. These wounds maintain a 1.0×10^6 AB5075 burden through day 10. (**b**) Progression from inoculated to dressed wounds, through bandaging, and vest application, (**c**) timeline of experiment, (**d**, **e**) 8 mm punches were used to collect biopsies for histopathology/RNA/protein/ELISA or CFU analysis

3. A 1.0–3.0 mL intravenous (IV) blood sample was taken for complete blood count (CBC) analyses.
4. On day −4, each pig received 25 mg/kg cyclophosphamide IV injection (*see* **Note 5**). The neutropenic agent cyclophosphamide (Baxter, Deerfield, IL) was dissolved in 0.9% sodium chloride injection solution (Hospira Inc., Lake Forest, IL) to obtain a final concentration of 100 mg/mL.
5. Pigs were fitted with a customized canvas vest for acclimation prior to wounding and bandaging.
6. On day 0, hair with an electric razor was clipped from the cervical to mid-lumbar dorsum, and the skin scrubbed with iodine solution followed by a chlorhexidine rinse.

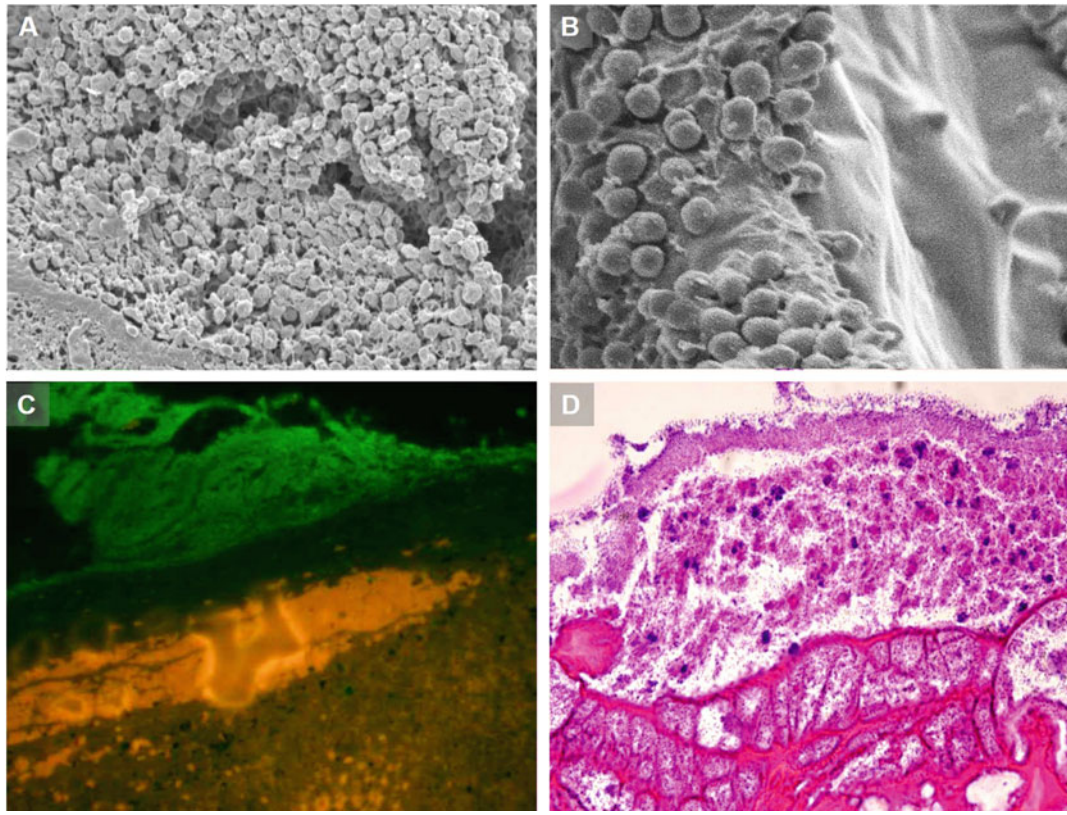

Fig. 4 Demonstration of *A. baumannii* infection in full-thickness excisional porcine wound. Scanning electron micrographs at (**a**) the wound bed and (**b**) the wound edge demonstrating biofilm formation. (**c**) Peptide nucleic acid fluorescent in situ hybridization of pig wound biopsy staining demonstrates the presence of bacteria (green stain). (**d**) Hematoxylin and eosin staining demonstrates the presence of bacteria (black dots) in the wound bed

7. A 12 mm disposable skin biopsy punch (Acuderm® Inc., Fort Lauderdale, FL) and surgical scissors were used to create 16 full-thickness skin wounds approximately 2 mm deep into the subcutaneous fat overlying thoracic and lumbar paraspinal musculature. Gauze is used to pack the wound to prevent excess bleeding until the inoculum is delivered (*see* **Note 7**).
8. On day 0, 50 μL containing 5.0×10^4 AB5075 cells in PBS suspension was pipetted into the wounds and allowed to absorb for 3 min. Bacteria were cultured in Lennox Luria-Bertani (LB) media (Becton, Dickinson and Co., Sparks, MD). 100 μL of AB5075 overnight culture was subcultured into 10 mL of LB and then grown at 37 °C and shaking at 250 rpm in a 250 mL Erlenmeyer flask. Cells were harvested when the culture grew to OD_{600} 0.7 (in mid-log growth phase). Cells were washed twice with sterile phosphate-buffered saline (PBS) and then resuspended in PBS at a concentration of 2.0×10^6 cells/mL.

9. After inoculation, a transparent dressing (Tegaderm™ Film 1622W, 3M Health Care, St. Paul, MN) was placed over each wound and secured with tissue adhesive (Vetbond™, 3M Animal Care, St. Paul, MN).
10. A 75 mcg/h fentanyl patch (Duragesic®, Janssen Pharmaceuticals, Inc., Titusville, NJ) was placed on the pig flank and replaced at subsequent sampling time points.
11. The pig torso was wrapped with bandaging tape (Vetrap™, 3M Animal Care, St. Paul, MN), and the canvas vest was reapplied.
12. Beginning on day 1 and at all subsequent time points (days 3, 7, and 10), four randomly assigned wound beds, one from each corner to account for anatomical variability, were sampled using a 4 mm disposable skin biopsy punch (Acuderm® Inc., Fort Lauderdale, FL). Biopsies were processed for histopathology, scanning electron microscopy, CFU analysis, ELISA, and qPCR.
13. A chlorhexidine-soaked gauze sponge was inserted into each sampled and debrided wound for 3 min to reduce bacterial load. Finally, wounds were closed with 3-0 polydioxanone suture material in a cruciate pattern.

4 Notes

1. *Topical vs. systemic treatments.* It should be noted that as companies and academic labs continue to develop new antibacterial treatments and toxicity is tested, many treatments, even at low doses, cannot be used systemically without causing adverse effects. This has spurred a secondary approach to use some of these treatments topically where the toxicity concern is less stringent. The benefit of the models presented is that both systemic and topical agents can be applied. It should also be noted that formulation is tremendously important. Understanding how a product performs as a gel, cream, or liquid and to what level the antibacterial reaches the wound bed can obviously affect outcomes. It is recommended that substantial testing in vitro with varying formulations be done before any animal experiments are considered.
2. *Bacterial strain selection.* For both models, any *A. baumannii* strain can likely be used, but the inoculum will need to be adjusted. For a strain more virulent than AB5075, cyclophosphamide may not be needed [26]. For less virulent strains, we found a log increase in the inoculum still resulted in a similar wound infection with some other strains (our unpublished data). However, this is certainly strain-dependent, and pilot

experiments will be needed to further assess the proper inoculating dose.

3. It should be noted that other agents can be used to dampen the innate immune response that still results in a prolonged infection. Two examples include the use of Ly-6G monoclonal antibody that blocks neutrophils specifically [24]. Another example is morphine that can dampen the overall inflammatory response [25]. The use of such agents depends on what is driving the experiment in the first place. For example, it may be better to use Ly-6G when components of the immune system are required for the antibacterial being tested such as immunomodulatory agents. Because cyclophosphamide has wide-ranging effects on the immune system, it may not be the best choice for that agent. In contrast, with standard antibiotic approaches that just attack the bacterium chemically, the use of cyclophosphamide might be more beneficial from a cost perspective.
4. *Mouse strain selection*. For the murine model, we routinely use BALB/c mice, but one should understand these are not the only mice that can be utilized when testing antibacterial agents. We initially selected BALB/c mice because they are skewed to a Th2 immune response, which appears to better suit Gram-negative infection that can be limited by the innate immune response (Th1 response) [27]. That said, we have also used male and female C57BL/6J mice, diabetic mice, and humanized mice with our protocol, each with varying outcomes and dependent on the agent being tested. However, female BALB/c mice have mild personalities and are easier to take care of, manipulate, and handle. They have less of tendency to bite when being held and less of a tendency to attack cage-mates or littermates during the duration of the experiment. It should also be noted that some strains of mice, such as A/J mice, are naturally susceptible to *A. baumannii* infection for various reasons and cyclophosphamide will not be needed [21]. The bottom line is that one should not feel limited by the mouse strain selection. Depending on the needs of the experiment, other strains of mice can certainly be used, but the wounding procedure and postsurgery care are the same.
5. For both of these models, the dosage of cyclophosphamide needs to be accurate. We explored both lesser and increased doses in pilot experiments with varying results. For example, when increased doses of cyclophosphamide were used, the animals remained neutropenic for a longer time, and the bacteria spread more easily into the bloodstream, and animals unfortunately perished. In contrast, when too little cyclophosphamide was used, the infection was cleared by the immune

system, and the wounds would close the same as uninfected wounds.

6. Enrichment and welfare for the animals is important. We have found that better care of the animals in both infection models results in better, more reproducible results. An example of this is the use of DietGel Recovery for the mice inside the cage after the procedure and inoculation. As the mice become infected, they become lethargic, and some animals have a tendency to nest rather than making repeated trips for food. Unfortunately, some animals can even succumb to infection under this stress, even though the infection itself is not a lethal dose. With the gel in the cage, the mice require less effort to obtain food, and this improves survivability and outcomes. Each mouse cage also includes a house for nesting and a toy. In the case of the porcine model, we enrich the animals' day-to-day life with toys and sometimes soothing music; therefore, at the time of the procedure, the animals are relaxed and easier to manipulate.
7. *Learning to make the wounds.* Making wounds with the punch biopsy instrument could be considered almost an art. Incisions made too deep result in wounds that significantly bleed and could overly harm the animal, and wounds made with the pressure being too light often did not result in an infection. Our staff are trained (and retrained) for many hours learning how to make the wounds with appropriate depth and with appropriate circular pressure. This included training with deceased animals before live animals were even attempted. Both models require a dedicated staff such as this. The murine model to effectively perform the procedure and treatment requires at least four people on the day of the surgery and at least two people for care and treatments, before and after the surgery. The porcine model requires even more dedicated staff as the animal is essentially treated as a human patient. We had at least two veterinarians monitoring and assisting with the pigs at all times.
8. For the murine wound model, single caging initially was required to prevent predation during the first few days of the experiment and after surgery and recovery. Again, as the animals become sick, they try to limit trips to food sources. Unfortunately, chewing on cage-mates' backs for food became a solution. Single caging prevented this during the initial stages of infection. After about a week (generally, after a few days of Tegaderm™ removal), animals can be housed together again as their immune systems fight the symptoms of infection, and animal health improves.

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Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted under approved animal protocols in an AAALAC-accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC publication, 2011 edition.

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