# Quantitative Comparison and Analysis of Species-Specific Wound Biofilm Virulence Using an In Vivo, Rabbit-Ear Model

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**BACKGROUND:** Although bacterial biofilm is recognized as an important contributor to chronic wound pathogenesis, differences in biofilm virulence between species have never been studied in vivo.

**STUDY DESIGN:** Dermal punch wounds in New Zealand white rabbit ears were inoculated with *Klebsiella pneumoniae*, *Staphylococcus aureus,* or *Pseudomonas aeruginosa*, or left uninfected as controls. In vivo biofilm was established and maintained using procedures from our previously published wound biofilm model. Virulence was assessed by measurement of histologic wound healing and host inflammatory mediators. Scanning electron microscopy (SEM) and bacterial counts verified biofilm viability. Extracellular polymeric substance (EPS)-deficient *P aeruginosa* was used for comparison.

**RESULTS:** SEM confirmed the presence of wound biofilm for each species. *P aeruginosa* biofilm-infected wounds showed significantly more healing impairment than uninfected, *K pneumoniae*, and *S aureus* ($p < 0.05$), while also triggering the largest host inflammatory response ($p < 0.05$). Extracellular polymeric substance-deficient *P aeruginosa* demonstrated a reduced impact on the same quantitative endpoints relative to its wild-type strain ($p < 0.05$).

**CONCLUSIONS:** Our novel analysis demonstrates that individual bacterial species possess distinct levels of biofilm virulence. Biofilm EPS may represent an integral part of their distinct pathogenicity. Rigorous examination of species-dependent differences in biofilm virulence is critical to developing specific therapeutics, while lending insight to the interactions within clinically relevant, polybacterial biofilms. (J Am Coll Surg 2012;215:388–399. © 2012 by the American College of Surgeons)

Bacterial biofilms, defined as a surface-adhered, complex community of aggregated bacteria within a matrix of extracellular polymeric substance (EPS), are increasingly being recognized as an integral component of chronic wound pathogenesis.[1-7] Given the enormous burden that these wounds place on patients and the health care system,[8-14] continued research aimed at delineating the mechanisms associated with wound biofilm development and maintenance remains critical. In particular, recent clinical studies have suggested that the predominant bacteria within a chronic wound can be one of several different species, and can often be present as polybacterial biofilm infections.[2,4,7,15] These findings are supplemented by studies that have demonstrated the formation of biofilm by different bacterial species, including *Staphylococcus aureus*,[16-18] *Pseudomonas aeruginosa*,[19-21] and *Staphyloccocus epidermidis*,[22-24] in a variety of in vivo model systems. The potential for wound biofilms to originate from different, or multiple, bacterial species further complicates our limited understanding, indicating a need to examine biofilm pathophysiology at a species-specific level.

Biofilm-phase bacteria can be distinguished from their free-floating, "planktonic" counterparts by their inherent defense and survival mechanisms. The biofilm EPS provides a physical barrier against inflammatory cell phagocytosis, while also po-

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**Abbreviations and Acronyms**

CFU = colony-forming unit
EPS = extracellular polymeric substance
POD = postoperative day
qRT-PCR = quantitative reverse transcription-polymerase chain reaction
SEM = scanning electron microscopy

tentially inhibiting the complement cascade and the activation and penetration of antibiotics.[22,25-28] Others have suggested that the shedding of planktonic bacteria and the maintenance of phenotypically distinct "persister" cells contribute to its sustainability and durability within the hostile environment of its host surface.[2,3] However, some protective mechanisms have been frequently linked to certain bacterial species more than others. Cell-to-cell signaling, termed *quorum-sensing*, has been implicated as a major component of *P aeruginosa* biofilm pathogenicity both in vitro and in vivo,[2,21,29,30] while it remains controversial in *S aureus*.[31] Meanwhile, several different regulatory molecules have been identified as important to the biofilm-forming ability of *S aureus*, including *sarA*,[32,33] *agr*,[34] and *cidA*.[35] As part of mediating resistance to neutrophils, *S epidermidis* biofilms use an intracellular adhesin to prevent phagocytosis, while *P aeruginosa* biofilms may diminish the neutrophils' oxidative potential[36,37] or lead to their rapid necrosis through rhamnolipid production.[38] Therefore, although the biofilm phenotype is common to most bacteria, individual bacterial species may use different mechanisms to achieve and maintain their presence within a wound.

Although the aforementioned differences in species-specific biofilms have been established, the end effects of each species' biofilm on wounds and their host, ie, virulence, are unclear. Clinical observation suggests that differences in biofilm virulence exist, as the appearance and severity of wounds can often be linked to one bacterial species over another based on experience. Unfortunately, these conclusions rely on anecdotal evidence rather than rigorous scientific experimentation. There remains no study in the literature, to date, that has evaluated and compared the species-specific virulence of different bacterial wound biofilms. Understanding species-dependent differences in biofilm pathogenicity may contribute to the development of specific, targeted biofilm therapeutics, while also lending insight to the interactions that occur within a polybacterial setting.

The goal of this study was to use our established, rabbit ear, wound biofilm model[16] to investigate whether there are differences in biofilm virulence across multiple bacterial species. Through comparison of the common wound pathogens *K pneumoniae*, *S aureus*, and *P aeruginosa*, we have discovered and attributed a distinct level of virulence to each species. In line with clinical observation, we demonstrated that *P aeruginosa* biofilm has the most significant effect on wound healing and the host inflammatory response. We investigated the mechanism of this pathogenicity using a mutant *P aeruginosa* strain. In doing so, we implicated the EPS as a critical contributor to Pseudomonas virulence, which has not been previously reported. With these results, we also validate the sensitivity of our in vivo system, establishing the model as a valuable and informative tool for translational biofilm research.

## METHODS

### Animals

Under an approved protocol by the Animal Care and Use Committee at Northwestern University, adult New Zealand white rabbits (3 to 6 months old, approximately 3 to 4 kg) were acclimated to standard housing and fed ad libitum. All animals were housed in individual cages under constant temperature and humidity with a 12-hour light-dark cycle. A total of 26 rabbits were used to complete this study.

### Bacterial species

Three separate bacterial species were used including individual strains of *K pneumoniae* (BAMC 07-18), *S aureus* (UAMS-1), and *P aeruginosa* (PAO1 and mutant strain *pelApslBCD*). The *K pneumoniae* strain BAMC 07-18 (kindly provided by LTC Clinton Murray of Brooke Army Medical Center, Fort Sam Houston, TX) was originally isolated from the wounds of an injured soldier that had returned from Iraq during the war. PAO1 was obtained from the laboratory of Dr Barbara H Iglewski (University of Rochester Medical Center). *P aeruginosa* mutant *pelApslBCD* was kindly provided by Dr Tim Tolker-Nielsen of the University of Copenhagen. The *pelApslBCD* mutant is a previously characterized double mutant of the *pel* and *psl* loci mutants, each of which causes deficiencies in the biosynthesis of polysaccharides that are part of *P aeruginosa* EPS.

To prepare bacterial culture each species was grown on specific agar plates (Hardy Diagnostics; BAMC 07-18 on blood agar, UAMS-1 on *S aureus* isolation agar, PAO1 and *pelApslBCD* on *P aeruginosa* isolation agar) overnight at 37°C. Each species was then subcultured at 37°C into 10 mL of tryptic soy broth (TSB; BAMC 07-18 and UAMS-1) or Luria broth (LB; *P aeruginosa* strains) and grown at 37°C until log-phase was achieved. Bacteria were harvested and washed in phosphate-buffered saline (PBS) once by centrifugation at 5,000 rpm for 5 minutes at 20°C. The resultant pellet was resuspended in PBS and an optical density at the 600-nm wavelength ($OD_{600}$) was measured. For each species, an $OD_{600}$ equivalent to $10^6$ colony-forming units (CFU)/$\mu$L was determined pre-empirically.