Polymicrobial Conditions Affect Antibiotic Susceptibility in Clinically Relevant Bacterial Species

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ABSTRACT

Chronic wounds, defined as those which remain open and inflamed for greater than six weeks, are a major area of clinical concern. Resulting in thousands of amputations per year and billions of dollars spent globally in treatment, chronic wounds are notoriously difficult to successfully treat. Two hallmarks of chronic wounds are that they are thought to harbor biofilm-associated bacteria and tend to be polymicrobial. While the research literature has repeatedly demonstrated the effects of biofilms on wound persistence and the changes to the efficacy of antibiotics, few studies have demonstrated what effect the polymicrobial condition has on the antibiotic tolerance of bacteria. To further explore this, four species of clinically relevant wound pathogens (*Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Staphylococcus aureus*, and *Enterococcus faecalis*) were tested in mono- and polymicrobial conditions using the current gold-standard clinical methods for determining antibiotic susceptibility. Noticeable differences in antibiotic tolerance were observed in the polymicrobial condition, including both increased and decreased susceptibility, depending on the antibiotic used. Our data demonstrate that the current clinical methods used for testing antibiotic susceptibility can generate results that are not representative of the infection environment, which may contribute to treatment failure and persistence of polymicrobial infections.

INTRODUCTION

CHRONIC WOUNDS and POLYMICROBIAL INFECTIONS

Chronic wounds are defined as persistent, slow healing wounds that remain open for an extended period of time. Chronic wounds do not progress through the normal stages of wound healing, as acute wounds do, and usually arrest in a state of chronic inflammation. Examples of chronic wounds include diabetic foot ulcers, pressure ulcers and surgical site infections. (Figure 1) (Wolcott *et al.*, 2010). Chronic wounds are commonly polymicrobial infections, consisting of numerous species of microorganisms. Polymicrobial infections have two major consequences of clinical concern. The first is the difficulty that exists in attempting to detect all of the microorganisms present using traditional pure-culture microbiology methods (Clinton *et al*., 2015). The second is the ability for microorganisms to synergize their activities, leading to more aggressive, difficult to treat, and virulent infections compared to mono-species (DeLeon *et al*., 2014, Smith *et al*., 2017). A group of bacteria of major clinical concern are known at the ESKAPE pathogens, which includes *Enterococcus faecalis*, *Staphylococcus aureus*, *Acinetobacter baumannii, and P. aeruginosa* (Santajit S. & Indrawattana N., 2016). These organisms are notoriously antibiotic resistant and are the one of the leading causes of nosocomial (hospital-acquired) infections in the US (Santajit S. & Indrawattana N., 2016). In chronic wounds, these organisms are some of the most common species identified, and they are much more likely to cause co-infections than mono-species infections (Dowd *et al*., 2008). One important distinction is between tolerance and resistance. Tolerance is an effect of differential gene expression, where metabolic patterns of bacteria prevent antibiotics from reaching or affecting their target sites, resulting in a transient change in antimicrobial efficacy. Resistance, due to genotypic changes from the acquisition of a gene, is a 'permanent' change in the bacterium's genetic makeup that allows it to neutralize an antibiotic.

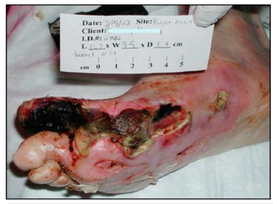

Figure 1: Diabetic foot ulcer
Photo courtesy of Dr. Randall Wolcott, MD, Southwest Regional Wound Care Center, Lubbock, TX

CLINICAL DIAGNOSTICS

Clinical microbiologists follows method guidelines set out by the Clinical and Laboratory Science Institute (CLSI) for microbial identification and antibiotic susceptibility testing (AST) of bacteria from patient samples (CLSI M100, CLSI M7). The current 'gold standard' method of determining antibiotic susceptibility is the Minimum Inhibitory Concentration (MIC) assay (Figure 2). The MIC is reported as the lowest concentration of antibiotic required to completely inhibit the growth of bacterial isolates and is used to determine the clinical dosage of antibiotic recommend to the physician. CLSI AST standards are determined on planktonic bacterial monocultures, despite research demonstrating that many infections, such as chronic wounds, exist as polymicrobial biofilms.

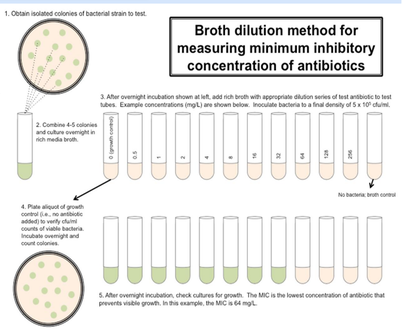

Figure 2: Minimum Inhibitory Concentration (MIC) method. Image from Labome

Researchers have extensively demonstrated that bacterial biofilm formation results in decreased efficacy of antibiotics, requiring up to 1000X concentration to be eradicated compared to planktonic ('free-living') infections (Mah *et al*., 2001). However, little is known about the effects of polymicrobial interactions on antibiotic susceptibility. Because the CLSI standards have no provision for polymicrobial or non-planktonic testing, this may result in antibiotic dosage recommendations that are not representative of what will be effective in the chronic would environment, which can result in persistent infections and worse health outcomes.

HYPOTHESIS

Polymicrobial communities will synergize to exhibit significantly different antibiotic susceptibility profiles compared to their monoculture counterparts

EXPERIMENTAL DESIGN

Our experimental design follows the CLSI M100 and M7 guidelines for determining AST in the clinical setting. CLSI-recommended quality control organisms were chosen because they have expected MIC values and are routinely tested in hospitals. The strains chosen were *S. aureus* ATCC® 29213 (SA), *E. faecalis* ATCC® 29212 (EF), *P. aeruginosa* ATCC® 27853 (PA), and *A. baumannii* ATCC® 19606 (AB). Clinical MICs are performed in Cation-Adjusted Mueller Hinton Broth (CAMHB) per CLSI M7.

MIC was determined on the individual bacteria first to establish that our experimental system generated results consistent with the CLSI M100 expected MIC values. Then, the four organisms (PA, SA, EF, AB) were mixed in a 1:1:1:1 polymicrobial planktonic suspension and an MIC panel was conducted. The MIC was determined by qualitative observed turbidity as determined by CLSI M100. However, changes to individual species' MIC cannot be observed by turbidity. Therefore, a secondary assay for viability was conducted, where the polymicrobial wells were extracted, diluted to the first order in 1XPBS, then plated on selective and differential media (Pseudomonas Isolation Agar for the recovery of PA, Mannitol Salt Agar for the recovery of SA, Bile Esculin Agar for the recovery of EF, and Leeds Agar for the recovery of AB) and the results were assessed between 18-24 hours of growth. Analysis of the results for statistical significance was omitted as many conditions had SD=0, and the observed differences in tolerance were notably different.

RESULTS

Polymicrobial Interactions Change Antimicrobial Susceptibility

(A) Gentamicin

Species	MIC Value (µg/mL)
P. aeruginosa	0.5±0†
S. aureus	0.5±0†
A. baumannii	4±0†
E. faecalis	8±0†
Polymicrobial	8±0

(B) Tetracycline

Species	MIC Value (µg/mL)
P. aeruginosa	32±0†
S. aureus	0.5±0†
A. baumannii	4±0†
E. faecalis	32±0†
Polymicrobial	128±0

(C) Penicillin

Species	MIC Value (µg/mL)
P. aeruginosa	>128±0
S. aureus	2±0†
A. baumannii	16±0
E. faecalis	4±0†
Polymicrobial	>128

(D) Ceftazidime

Species	MIC Value (µg/mL)
P. aeruginosa	2±0†
S. aureus	16±0†
A. baumannii	1±0†
E. faecalis	>128±0
Polymicrobial	16±0

Figure 3 - Comparison of Minimum Inhibitory Concentrations (MIC) in Mono- and Polymicrobial Conditions. Monomicrobial versus Polymicrobial MIC results for gentamicin (A), tetracycline (B), penicillin (C), and ceftazidime (D). † denotes that the value is within the published guidelines from the Clinical Laboratory Science Institute's M7 and M100 manuals for MIC breakpoints ((CLSI, 2018a, 2018b)). MIC values greater than 128 µg/mL have been calculated as that number. CLSI does not publish established breakpoint values for *P. aeruginosa* and *A. baumanii* when treated with penicillin and for *E. faecalis* when treated with ceftazidime. n=3.

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(A) Penicillin

Species	Individual Viability Value (µg/mL)	Polymicrobial Viability Value (µg/mL)
P. aeruginosa	>128±0	>128±0
S. aureus	2±0†	128±0
A. baumannii	16±0	>128±0
E. faecalis	4±0†	4±0

(B) Ceftazidime

Species	Individual Viability Value (µg/mL)	Polymicrobial Viability Value (µg/mL)
P. aeruginosa	>128±0†	>128±0
S. aureus	96±55.43†	32±27.71
A. baumannii	37.33±24.44†	106.67±37.0
E. faecalis	>128±0	>128±0

Figure 4 - Comparison of Viability in Mono- and Polymicrobial Conditions. Monomicrobial versus Polymicrobial Viability results for penicillin (A) and ceftazidime (B). † denotes that the value is within the published guidelines from the Clinical Laboratory Science Institute's M7 and M100 manuals for MIC breakpoints ((CLSI, 2018a, 2018b)). Viability values greater than 128 µg/mL have been calculated as that number. CLSI does not publish established breakpoint values for *P. aeruginosa* and *A. baumanii* when treated with penicillin and for *E. faecalis* when treated with ceftazidime. n=3.

SIGNIFICANCE and FUTURE DIRECTIONS

Our data across the two methods - MIC and viability - demonstrate several notable results.

For the MIC experiments measured by turbidity, gentamicin, tobramycin, penicillin, and doxycycline did not show a difference of the observable MIC in the polymicrobial condition over the given values for the individual. In other words, the range of possible MIC values of the individual condition overlapped with that of the polymicrobial. Because of this, and because the samples were assessed visually, it is not possible to determine the contribution of each individual species to the turbidity observed in the polymicrobial condition. Two notable differences in MIC were observed in the polymicrobial condition, however, for ceftazidime and tetracycline. In tetracycline, a decrease in susceptibility was observed, which correlates to a decreased antibiotic efficacy, presumably via tolerance. As the polymicrobial MIC is substantially greater than any of the component individual MICs, it is not possible to determine which bacterial species' tolerance was increased, or which combination of those species was affected. In ceftazidime, however, an opposite effect was observed, where a sensitization interaction occurred for *E. faecalis*.

For the viability experiments, bacteria treated with penicillin and ceftazidime both showed notable differences in antibiotic susceptibility in the polymicrobial condition. When treated with penicillin in the polymicrobial environment, both *S. aureus* and *A. baumanii* showed notable increases in tolerance to the antibiotic challenge, but when treated with ceftazidime, *S. aureus* demonstrated a decreased tolerance to the antibiotics and *A. baumanii* showed an increased in tolerance to the compound.

Given that our results are consistent with both the existing literature around the polymicrobial effect on antibiotic susceptibility and uses the current clinical model, these data demonstrate that there exists a gap in the clinical diagnostic schema for determining antimicrobial susceptibility of polymicrobial infections. As it has also been repeatedly demonstrated in the literature that synergistic interactions among bacteria within a wound produce more negative outcomes, and that chronic wounds have repeatedly been shown to harbor polymicrobial infections, our data is consistent both internally and externally with the observations found in clinical practice. Since both tolerance and resistance can play important roles in the success of infection treatment, and as our data demonstrates, tolerance alone can notably change the susceptibility of bacteria to antibiotic treatments, it is critical that the clinical models be adapted to allow for the presence of polymicrobial cultures during the assessment process. This change could potentially result in more accurate assessments of antibiotic susceptibility across the clinical spectrum, and may be of particular benefit to the treatment of chronic wounds. In addition, as both polymicrobial colonies and biofilms can exist simultaneously, the effects seen in these studies might be compounded when combined in the clinical setting, and more research needs to be done to understand both the cumulative effect of those conditions in the clinical wound setting and how the entirety of the microbial environment can be taken into account when considering antimicrobial susceptibility in clinical diagnostic procedures. As a further note, though the rise of sequencing technologies in the clinical laboratory is no doubt of great value for clinical microbiologists and provides valuable data in the diagnostic process (particularly for microbial identification), because the above data assesses transient changes to antibiotic tolerance rather than antibiotic resistance, methods such as 16S next-generation sequencing (NGS) or rapid qPCR will not be able to determine these changes in tolerance, since those technologies rely on and assess for the presence of antibiotic resistance genes.

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