NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

EVALUATION OF ANTIMICROBIAL PEPTIDES AGAINST PSEUDOMONAS AERUGINOSA IN VARIOUS MEDIA

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DECLARATION OF INTEREST

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ABBREVIATIONS

AMP	Antimicrobial peptide
BM2	Basal media two
BAMC MBRL	Brooke Army Medical Center Molecular Biology Research Laboratory
CFU	Colony forming units
HGEP	Human gingival epithelium progenitors
µg	Microgram
M9	M9 salts minimal media
$MBEC_{90}$	Minimum biofilm eradication concentration, 90%
MDR	Multidrug resistant
MH	Mueller-Hinton
MIC_{90}	Minimum inhibitory concentration, 90%
ml	Milliliter
mM	Millimolar
PBS	Phosphate buffered saline
OD_{570}	Optical density to 570 nanometer wavelength light
OD_{600}	Optical density to 600 nanometer wavelength light
TSB	Tryptic soy broth
WST-1	Water soluble tetrazolium salt 1

EXECUTIVE SUMMARY

Background: Multidrug resistant (MDR) bacteria, and nosocomial infections that arise from them, are increasingly common, with military personnel being at even greater risk than the general population. Unfortunately, few novel antibiotics are currently in development to combat them. The use of antimicrobial peptides (AMPs) is one new avenue currently under development to address the global need for new antibacterial therapies. However, as experiments use varying growth conditions, target species or strains, and doses, research consensus on AMP efficacy is limited.

Objective: The purpose of this study was to evaluate the effect of bacterial growth conditions on AMP antibacterial activity, and set a reasonable standard for objective comparison between AMPs. Three AMPs were selected: AMP 1018, KSL-W, and K_6L_9, and tested against *Pseudomonas aeruginosa*, an opportunistic pathogen of international concern. AMP selection was based on their peptide size, mode of action, efficacy, cytotoxicity, and to include AMPs derived from a diversity of organisms. This evaluation was designed to balance antibacterial and antibiofilm activity against cytotoxicity levels.

Methods: Standardized antibacterial activity assays were conducted with the three selected AMPs against four varieties of *P. aeruginosa*; two laboratory strains and two MDR clinical isolates. Bacteria were exposed to various growth conditions; two different minimal and two different complete media. AMPs were also tested for antibiofilm activity and cytotoxicity in human gingival epithelium progenitor cells.

Results: Antibacterial and antibiofilm efficacies varied by over 100-fold with target bacterial strain and growth conditions. AMP 1018 disrupted biofilms at low concentrations while K_6L_9 was effective against both laboratory strains and biofilms. KSL-W showed generally better antibacterial activity but was less effective in disrupting biofilms than 1018. Culture medium had a larger impact on AMP efficacy than strain identity, including MDR status.

Conclusions: Changes in bacterial growth conditions have large effects on AMP efficacy. Standardized protocols and growth conditions can minimize these effects and enable reasonable comparison of AMP antibacterial or antibiofilm effects across AMPs and between research groups.

INTRODUCTION

The emergence and increasing prevalence of bacterial strains resistant to available antibiotics poses a serious threat to world health. Formerly routine infections have become increasingly challenging to treat clinically. Even alcohol-based surface disinfectants commonly found in hospitals and clinics are now tolerated by some bacterial pathogens (Pidot et al., 2018). Recently, the World Health Organization published a report indicating that antimicrobial resistance threatens the effective prevention and treatment of bacterial infections (WHO, 2014). Additionally, the Centers for Disease Control and Prevention reported that antibiotic resistance leads to two million serious infections and 23,000 deaths each year, and adds $20 billion in excess direct health care costs beyond the $35 billion in lost productivity per year (Mackowiak, 2013). To combat the rise of antibiotic resistant bacteria, the White House released a comprehensive critical action plan (The White House, 2015). Bacterial strains resistant to one antibiotic often go on to develop resistances to others, becoming multidrug resistant (MDR) and making them generally more difficult to treat. Some strains eventually acquire resistance to antibiotics or drugs with diverse modes of action (Nikaido, 2009). These strains can persist in healthcare environments, and the infections caused are inherently difficult to treat. MDR bacterial infections have become an increasing concern for troops at home and abroad over the last decade (Hospenthal et al., 2011). MDR *E. coli* colonization was 5.5 times more likely in deployed troops than in personnel in the U.S. (Vento et al., 2013). Taking action to control the rising rates of MDR infections, the U.S. Department of Defense launched the Antimicrobial Resistance Monitoring and Research Program in 2009, which rapidly identifies and monitors emerging MDR pathogens of public health concern (Lesho et al., 2014).

Developing novel antibiotics is typically not lucrative, and therefore the number of new antibiotics entering the market from pharmaceutical companies has declined each year, even as MDR infections are still on the rise (Spellberg et al., 2008). Developing these new drugs involves a great deal of time and expense, but the Food and Drug Administration ultimately approves only 20% of drugs that go through initial human trials (Hay, Thomas, Craighead, Economides, & Rosenthal, 2014). Thus, the challenges of developing new antibiotics for highly resistant bacterial infections has narrowed the drug pipeline to only a few new antibiotics each year (Spellberg et al., 2008). Due to the widening gap between numbers of newly approved drugs and MDR infections, there is a need to identify and develop novel, low cost, safe, and effective broad-spectrum antimicrobials to treat infections caused by emergent MDR pathogens, reducing both military and civilian morbidity and mortality. Fortunately, various novel treatments and therapeutics are emerging which will address this looming antibiotic resistance crisis (Schooley et al., 2017).

Using antimicrobial peptides (AMPs) is one such avenue for disrupting MDR infections. AMPs are capable of targeting and eliminating many types of pathogens, making them an attractive alternative for treating biofilms (Akers et al., 2014) and MDR bacterial infections (Alves & Olívia Pereira, 2014; Cruz, Ortiz, Guzmán, Fernández-Lafuente, & Torres, 2014). Due to their unique multidirectional modes of action and broad-spectrum activities, AMPs are less likely to induce bacterial resistance than conventional antibiotics (Chan, Prenner, & Vogel, 2006), which usually target a single enzymatic reaction (Fjell, Hiss, Hancock, & Schneider, 2011). However, the lack of standardized evaluation methods to test the efficacy of AMPs, and inconsistent efficacy data from varied testing methods, make relative comparisons of newly developed AMPs challenging.

A variety of media types have been used when testing AMPs *in vitro* (Harrison, Abdel-Rahman, Miller, & Strong, 2014; Porat, Marynka, Tam, Steinberg, & Mor, 2006), including complete media; tryptic soy broth (TSB) (Czihal et al., 2012; Knappe et al., 2010) or Mueller-Hinton (MH) broth

(Williams, Sroussi, Abercrombie, Leung, & Marucha, 2012; Zairi et al., 2014); minimal media (Andresen, Tenson, & Hauryliuk, 2016; de la Fuente-Nunez, Reffuveille, Haney, Straus, & Hancock, 2014); and phosphate-buffered saline (PBS) (Chen et al., 2013). While complete media contain all of the nutrients an organism requires for exponential growth, minimal media contain less, and buffers contain none at all. This variability in nutrient content makes side-by-side comparison of these studies problematic.

To properly evaluate novel compounds for comparison and potential antimicrobial use, standardized assay conditions must be established. Previous research shows that bacterial susceptibility to antibiotics varies extensively with media and growth conditions (Colquhoun, Wozniak, & Dunman, 2015; Jackson, Fedorka-Cray, Jackson-Hall, & Hiott, 2005; Koch & Gross, 1979). While growth media and conditions are tightly controlled during clinical trials, there are no guidelines or standards for early-stage AMP research.

To that end, we tested three AMPs: peptide 1018 (Andresen et al., 2016; de la Fuente-Nunez et al., 2014), KSL-W (Dixon, Karimi-Naser, Darveau, & Leung, 2008), and K_6L_9 (Makovitzki, Fink, & Shai, 2009) for antibacterial effects against *P. aeruginosa* in two complete and two minimal media growth conditions. Peptide 1018 was selected for its potential as a broad-spectrum antimicrobial (Andresen et al., 2016; de la Fuente-Nunez et al., 2014). The others were selected for their previously observed potency against pathogenic bacteria (Dixon et al., 2008; Makovitzki et al., 2009). We tested these AMPs against two laboratory strains, the common laboratory strain PAO1 (Stover et al., 2000) and a strain with additional virulence genes, PA14 (Mikkelsen, McMullan, & Filloux, 2011), as well as against two clinical isolates highly resistant to multiple antibiotics, 105734 and 105765 (from the Brooke Army Medical Center Molecular Biology Research Laboratory). These clinical isolates display increased resistance to a wide range of antibiotics, including ciprofloxacin and levofloxacin (unpublished data, BAMC MBRL).

We also tested each AMP against established *P. aeruginosa* biofilms grown in complete media, which may recapitulate *in vivo* conditions better than tests in planktonic liquid cultures. Finally, we screened each AMP for cytotoxicity in human gingival epithelium progenitor cells. This cell type was selected as a model because they reside within the human oral cavity, a highly complex multispecies and potentially pathogenic ecosystem (Dickinson et al., 2012).

MATERIALS AND METHODS

Materials

Antimicrobial peptides. Online searches and selection of novel AMPs were conducted through the Antimicrobial Peptide Database 3 and the Database of Antimicrobial Activity and Structure of Peptides. Selections were made based on their peptide size, mode of action, efficacy, cytotoxicity, and to include AMPs from multiple original sources. Peptide 1018 (VRLIVAVRIWRR; (de la Fuente-Nunez et al., 2014)), KSL-W (KKVVFWVKFK; (Dickinson et al., 2012)), and K_6L_9 (LKLLKKLLKKLLKLL; (Makovitzki et al., 2009)) were selected and were synthesized by Biomatik Inc. (Wilmington, DE) and Genemed Synthesis Inc. (San Antonio, TX). AMPs were stored at -80°C until use.

Bacterial strains and media. P. aeruginosa laboratory strains PAO1 (Stover et al., 2000) and PA14 (Mikkelsen et al., 2011), and clinical isolates 105734 and 105765, were grown at 37°C in 5% CO_2 with shaking at 250 rpm unless otherwise noted. Cultures were grown in basal media two (BM2, 62 mM KPO_4 pH 7.0, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 µM $FeSO_4$, 0.4% glucose), M9 salts minimal media (M9, 1 × M9 Salts pH 7.4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% glucose), tryptic soy broth (pH 7.3, 1.7%

tryptone, 0.3% phytone, 85.6 mM NaCl, 0.25% K_2HPO_4, 0.25% glucose; Becton Dickinson, Franklin Lakes, NJ), or 7.4 Mueller-Hinton broth (pH 7.4, 0.2% beef infusion solids, 1.75% casein hydrolysate, 0.15% starch; Becton Dickinson, Franklin Lakes, NJ).

Procedures

Antibacterial (planktonic) activity assays. Minimum inhibitory concentration (MIC) assays were conducted as described previously (Andresen et al., 2016; Knappe et al., 2010). Briefly, cells were grown overnight in BM2, M9, TSB, or MH media, then diluted to 10^5 to 10^6 colony forming units (CFU) per milliliter before being deposited into each well of a 96-well flat-bottom non-treated plate (Thomas Scientific, Swedesboro, NJ). Plates were incubated 18 hours and each well's optical density at 600 nm (OD_{600}) was recorded. A background value calculated from the mean OD_{600} of all media-only wells in each plate was subtracted from each OD_{600} measurement. Background-subtracted OD_{600} measurements were then pooled into means for each combination of AMP concentration, growth medium, and bacterial strain. The mean for each combination of conditions was divided by the mean OD_{600} for media and cells without AMPs, and this value was subtracted from one to give the mean proportion of bacteria in each condition relative to the appropriate control. Mean relative values were multiplied by 100 to compute the percent inhibition. MIC_{90} was defined as the lowest peptide concentration calculated to have less than 10% of the mean relative value of bacteria in wells without AMPs. Standard deviation values were scaled to the Y axis by multiplication by 100. When calculating mean $MIC_{90,}$ any individual replicates which displayed MIC_{90} greater than 256 µg/ml were recorded as MIC_{90} equal to 512 µg/ml.

Antibiofilm activity assays. Antibiofilm assays were similar to antibacterial assays described above, and have been described previously (O'Toole, 2011). 96-well plates were inoculated with bacteria in yeast-peptone broth (1% yeast extract, 2% peptone plus 1 g/l glucose) and incubated at 37°C without shaking for 18 hours to establish biofilms. After the biofilms formed, spent media was aspirated and wells were gently washed once with one volume of PBS. Complete or minimal media with the appropriate concentration of AMP was gently added to the wells, and plates were incubated for three hours without shaking. After incubation, the media was gently removed, wells were rinsed with PBS, and biofilms stained with 0.01% crystal violet at room temperature. After 30 minutes, the staining solution was removed and wells were rinsed once with PBS, plates were dried for 15 minutes in a fume hood, then 150 µl of 33% acetic acid was added to each well to resuspend the crystal violet that had stained the biofilms. Acetic acid was transferred to a clean plate, and the OD_{570} of each well was recorded.

Antibiofilm assay results were analyzed as MIC results, as described above, but percent biofilm remaining was calculated, rather than percent inhibition. Background was subtracted from OD_{570} values for each experimental well, each resulting value was expressed as a proportion of the mean OD_{570} of the appropriate 96-well plate's control wells without AMP, and multiplied by 100. For calculating a 90% minimum biofilm eradication concentration ($MBEC_{90}$), any individual replicates which displayed $MBEC_{90}$ greater than 128 µg/ml were recorded as $MBEC_{90}$ equal to 256 µg/ml. Between three and 18 replicates were conducted for each combination of bacterial strain, AMP, and AMP concentration.

Statistical Analysis. All MIC and MBEC experiments were conducted at least in triplicate. Each OD_{600} and OD_{570} for MIC and MBEC was measured from three separate wells. Due to limited cell availability, cytotoxicity tests were conducted at least in duplicate. Each OD_{440} and OD_{600} for WST-1 test was measured from three separate wells. The Mann-Whitney test was used to determine the level of significance for MIC_{90} and $MBEC_{90}$ data.

Cytotoxicity assays. Human gingival epithelium progenitor (HGEP, ZenBio Inc., Research Triangle Park, NC) cells were grown in CellnTec-Prime (CellnTec, Bern, Switzerland), a complete media for mammalian cell culture, at 37°C and 5% CO_2 at a concentration of 1.25×10^4 cells per well in a 96-well plate. After 24 hours, cells were washed with PBS, fresh media containing the appropriate concentration of AMP was added, and plates were incubated. After a further 24 hours, the water soluble tetrazolium salt one cell proliferation reagent (WST-1, Roche Diagnostics GmbH; Mannhein, Germany) was used to determine HGEP cell viability. Media was aspirated and replenished before 10 µl WST-1 reagent added to each well. Triton X-100 was used to induce cell death as a positive control. After plates were incubated for two hours, cell viability was inferred from the difference between OD_{440} and OD_{600}. Between two and six replicates were conducted for each set of experimental conditions, and the differences between OD_{440} and OD_{600} for each set were pooled to calculate mean percent cell survival and standard deviation.

RESULTS

We tested the antibacterial effect of each antimicrobial peptide against planktonic bacterial cells (Figures 1-3). All AMPs were less effective against cells grown in complete media (Figures 1-3 A-B) than against cells grown in minimal media (Figures 1-3 C-D). This pattern was especially clear for KSL-W, which had no mean MIC_{90} values greater than 16 µg/ml in minimal media while most mean MIC_{90} values in complete media were over 16 µg/ml (Figure 2 E), though incubation with 1018 and K_6L_9 yielded similar effects (Figures 1 E, 3 E). Mean MIC_{90} values for 1018 in laboratory strains (151.8 µg/ml) versus clinical isolates were similar (128.5 µg/ml), while those for minimal media (6.2 µg/ml) were much lower than those for complete media (285.9 µg/ml, Figure 1 E). The same was true for KSL-W (60.9 µg/ml for laboratory strains, 20.6 µg/ml for clinical isolates, 9.9 µg/ml for minimal media, 79.2 µg/ml for complete media, Figure 2 E) and K_6L_9 (40.7 µg/ml for laboratory strains, 52.1 µg/ml for clinical isolates, 9.0 µg/ml for minimal media, 86.1 µg/ml for complete media, Figure 3 E).

Peptide 1018 was significantly less effective against cultures grown in TSB than all other AMPs ($p < 0.0001$), though it did inhibit all strains when they were grown in MH (Figure 1 E). The antibacterial properties of K_6L_9 are qualitatively similar. K_6L_9 was also significantly more effective at inhibiting bacterial growth when cells were grown in minimal media than when they were grown in complete media ($p < 0.0001$, Figure 3 E). K_6L_9 was significantly more effective than 1018 against cells grown in TSB ($p < 0.0001$, Figures 1 E and 3 E), but all AMPs tested were significantly more potent against cells grown in minimal media than cells grown in complete media (1018: $p < 0.0001$, Figure 1 E; KSL-W: $p < 0.0001$, Figure 2 E). KSL-W inhibited both clinical strains when they were grown in MH, though it was most effective against the laboratory strain PA14 when grown in minimal media ($p < 0.0001$, Figure 2 E).

Each AMP was also effective at disrupting established biofilms when used in high concentrations (Figure 4). At low concentrations, between 1 and 4 µg/ml, AMP antibiofilm effects varied, but above 8 µg/ml for K_6L_9, 16 µg/ml for 1018, and 32 µg/ml for KSL-W each AMP eradicated most or all of the biofilm (Figure 4 A-C). Each AMP had lower mean $MBEC_{90}$ against clinical isolates (6.1 µg/ml for 1018, 29.3 µg/ml for KSL-W, 3.4 µg/ml for K_6L_9) than against laboratory strains (22.1 µg/ml for 1018, 226.7 µg/ml for KSL-W, 8.0 µg/ml for K_6L_9, Figure 4 D).

Finally, all AMPs were tested for cytotoxicity in HGEPs. Peptide 1018 and KSL-W were well-tolerated by the HGEPs, indicated by roughly 100% survival from 8 to 32 µg/ml, but at concentrations above 32 µg/ml we did observe cytotoxic effects (Figure 5). At 0.5 µg K_6L_9/ml HGEP survival was varied, but as concentrations increased above 1 µg/ml their survival rates dropped rapidly (Figure 5).

DISCUSSION

Antimicrobial peptides are typically short, amphipathic, cationic peptides, but variations in their primary and secondary structures (Hancock & Sahl, 2006) underscore their diverse effects including: inhibiting bacterial growth and biofilm formation (Andresen et al., 2016; Anunthawan, de la Fuente-Nunez, Hancock, & Klaynongsruang, 2015; Leung et al., 2005), eradicating established biofilms (de la Fuente-Nunez et al., 2014; Leung et al., 2005), blocking bacterial endotoxicity (Dixon et al., 2008), reducing cancerous tumors (Delouces & Di, 2017), and shuttling foreign DNA across cell membranes (Zhang et al., 2013). By acting on the bacterial membrane or by traversing it, AMPs can be used against multiple species (Andresen et al., 2016; de la Fuente-Nunez et al., 2014). This broad spectrum of activity makes AMPs appealing targets for combating antibacterial resistant or MDR bacterial infections, and their nonspecific modes of action make them less likely to induce bacterial resistance (Chan et al., 2006). Unfortunately, many distinct methods have been used to characterize AMP effects, making direct comparison between studies difficult.

We tested three AMPs for bacterial inhibition and biofilm eradication effects against four different strains of *P. aeruginosa* of varying antibiotic resistance: the common laboratory strain PAO1 (Stover et al., 2000), the more virulent laboratory strain PA14 (Mikkelsen et al., 2011), and two clinical isolates, 105734 and 105765, from the Military Health System (MHS). The clinical isolates 105734 and 105765 are both resistant to many conventional antibiotics, including ciprofloxacin and levofloxacin. AMP 1018 (Andresen et al., 2016; de la Fuente-Nunez et al., 2014), KSL-W (Dickinson et al., 2012), and K_6L_9 (Makovitzki et al., 2009) were proportionally more effective antibacterial (Figures 1-3) or antibiofilm (Figure 4) agents with increased dosages.

At high concentrations, each AMP tested did inhibit bacterial growth (Figures 1-3), though some were more effective against certain strains or under specific bacterial growth conditions. Each strain or isolate tested was generally more resistant to AMPs when cultured in complete media. There were up to 100-fold differences between AMP efficacies in minimal vs. complete media (Figures 1-3). We did not observe similarly large differences between efficacies against clinical isolates vs. laboratory strains (Figures 1-3). AMP efficacy against planktonic bacteria was more dependent on media conditions than the type (laboratory strain vs. clinical isolate) of bacteria tested (Figures 1-4).

It is encouraging that AMPs are effective against pathogenic, MDR bacterial biofilms. Indeed, each AMP tested had a lower mean $MBEC_{90}$ against clinical isolates than against laboratory strains (Figure 4D). The clinical *P. aeruginosa* strains used here are resistant to many drugs, including ciprofloxacin and levofloxacin, but against these MDR bacterial strains AMPs inhibited growth and eradicated biofilms. Rather than targeting and inhibiting a single enzyme or biochemical process as antibiotics typically do (Fjell et al., 2011), AMPs have broad anti-membrane or transmembrane effects (de la Fuente-Nunez et al., 2014; Hancock & Sahl, 2006). This makes it less likely that a bacterial strain will acquire resistance (Chan et al., 2006), and makes AMPs appealing targets for further development as therapeutics.

The strong effect of bacterial growth conditions on AMP efficacy, however, indicates that selecting the appropriate AMP-based treatment to counter a specific pathogen may depend on the *in vivo* environment of the infected tissue or wound. When testing AMPs for future therapeutic use, experimental conditions must also be chosen carefully.

Controlling for all the *in vivo* environmental factors present *in vitro* can be quite difficult. *In vitro* experiments generally do not recapitulate the nutrients, nutrient flow, surfaces, or microorganisms found

in vivo. The media effects we observed in our simplified *in vitro* methods will surely be compounded by steep chemical gradients (especially O_2 and CO_2), host-pathogen interactions, host immune responses, fluid flows, bacterial gene regulation changes and virulence factors, and biofilm presence and structure encountered *in vivo* (Roberts, Kragh, Bjarnsholt, & Diggle, 2015).

The best growth conditions for *in vitro* AMP experiments will either be tailored to the experiment's purpose or to the relevant tissue. Experiments developing AMP-based therapeutics may utilize *in vitro* conditions which best mimic *in vivo* conditions. Investigators seeking an AMP-based therapy to combat *P. aeruginosa* in cystic fibrosis may use artificial sputum in a microaerobic environment similar to the one found in expectorated sputum (Kolpen et al., 2010), but even this complex model would exclude the host immune system response, among other factors.

In vitro conditions will never fully reproduce those found *in vivo*, but they can be optimized. Our results show that AMP efficacies tend to be lower in complete media than in minimal media, where they are more variable. This variability is useful when comparing AMPs with one another. Artificially increased efficacies from experiments with minimal media can reveal differences in AMP efficacy that would require much higher AMP concentrations to observe in complete media.

Experiments and tests with AMPs in minimal media also benefit from the chemically defined nature of most minimal media recipes. Unlike complete media, which usually include components derived from animal tissue or serum that can vary from batch to batch, minimal media typically contain only chemically defined ingredients. This reduction in inter-batch variability can make comparing results simpler and more straightforward.

Finally, the end-use of a novel drug, or AMP, must be taken into account. For example, testing antibiotics targeted to intestinal pathogens in a media that replicates whole blood may yield less useful results than testing in media more appropriate to the relevant tissue. While the targeted pathogens may grow better in blood-based media, the results from this study would be less useful than one with growth conditions that mimic the gut. Based on our results, bacterial growth conditions have large effects on antibacterial efficacies which should not be discounted.

There is a strong need for one therapy that can both treat presently resistant strains and prevent new ones from emerging. Identifying and using broad spectrum AMPs to treat and prevent resistant infections will begin to close the resistance gap that puts the U.S. military population at risk. Therapies leveraging AMPs that can inhibit bacterial growth and eradicate MDR pathogenic biofilms, like 1018, KSL-W, and K_6L_9 do, will be very useful as antibiotic efficacies continue to decline, MDR bacteria spread, and nosocomial infections persist.

MILITARY SIGNIFICANCE

Multidrug resistant bacterial infections are increasingly common in the general population (WHO, 2014), but the deployed military population is at even greater risk (Vento et al., 2013). MDR *P. aeruginosa* infections in the MHS are also a concern; *P. aeruginosa*'s susceptibility to doripenem, imipenem, and ticarcillin/clavulanic acid has dropped significantly and continues to decrease (Gierhart & Chukwuma, 2017). Two major antibiotics for *P. aeruginosa* infection–ciprofloxacin and levofloxacin–showed less than 90% efficacy in the MHS in 2015. Additionally, a significant correlation between biofilm production and the persistence of MDR wound infections was reported in military trauma patients (Akers et al., 2014). The increased risk for U.S. military personnel, plus the anticipated dearth of effective antibiotics, indicates our military will bear the brunt of a post-antibiotic era. Antimicrobial peptides are

one possible solution to this looming crisis, but inter-group discrepancies in experimental design and growth conditions make absolute comparisons of discrete AMP studies impossible.

We investigated the antibacterial and antibiofilm properties of three AMPs at various concentrations, across four growth conditions, with standardized testing protocols. We also tested the AMPs against MDR clinical isolates and against two typical laboratory strains. Finally, we checked for cytotoxicity in HGEPs grown in a complete, mammalian cell culture media supplemented with varying concentrations of each AMP.

Our findings show that AMP efficacies are more dependent upon bacterial growth conditions than on multidrug resistant status. *P. aeruginosa* was more resistant to all AMPs tested when cultured in complete media than when cultured in the more stringent conditions of minimal media. Each of the AMPs we tested could inhibit bacterial growth and eradicate biofilms of MDR *P. aeruginosa*, which ciprofloxacin and levofloxacin, two antibiotics used in the MHS, could not. These results indicate that AMPs have great promise as novel antimicrobials or antibiotics, especially against MDR strains and nosocomial infections, but that conditions used to test them must be held constant. Failure to introduce and adhere to more systematic growth conditions or testing protocols will continue to hamper AMP research and slow down the discovery and development of new treatments for these problematic infections in the MHS.

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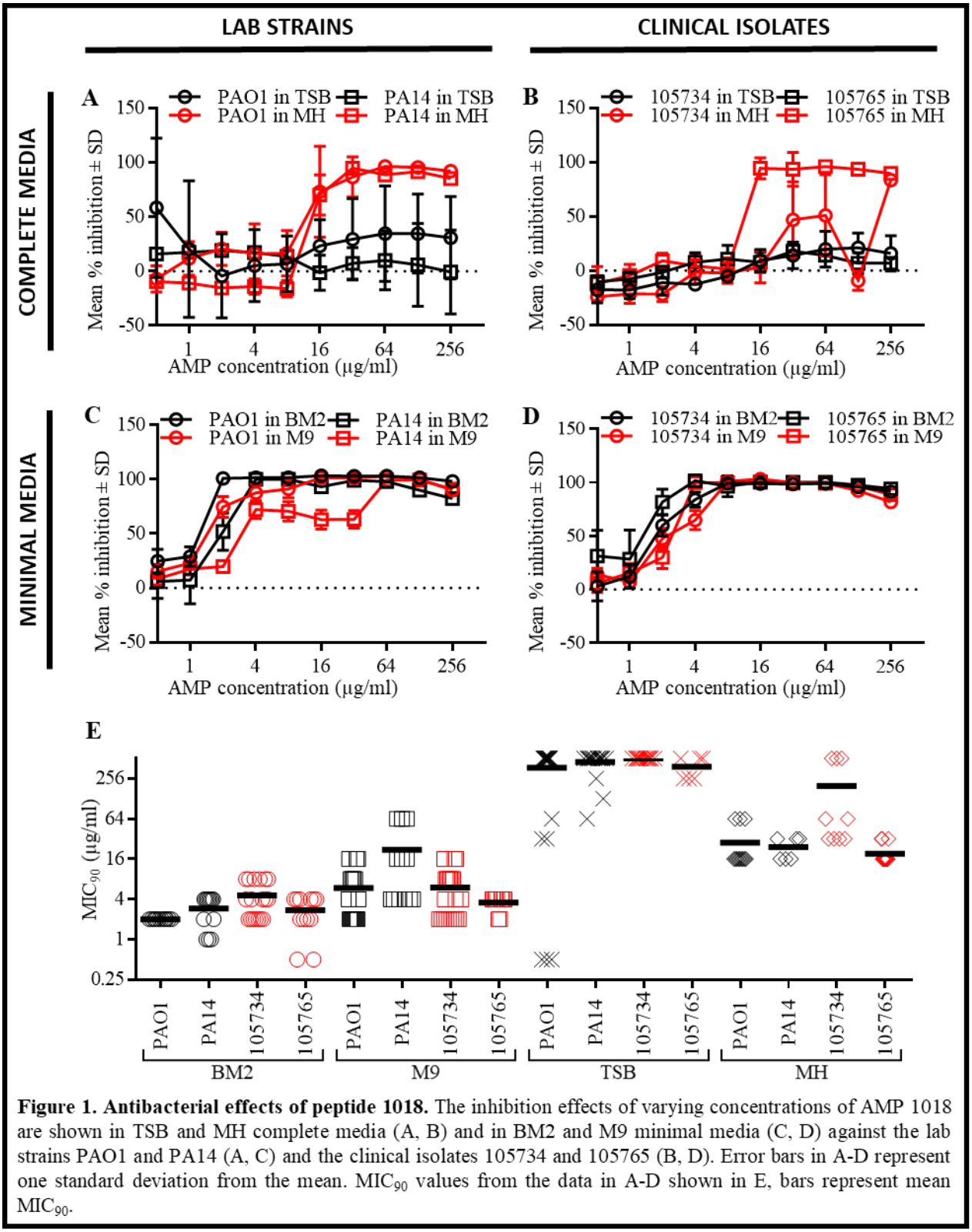

Figure 1. Antibacterial effects of peptide 1018. The inhibition effects of varying concentrations of AMP 1018 are shown in TSB and MH complete media (A, B) and in BM2 and M9 minimal media (C, D) against the lab strains PAO1 and PA14 (A, C) and the clinical isolates 105734 and 105765 (B, D). Error bars in A-D represent one standard deviation from the mean. MIC_{90} values from the data in A-D shown in E, bars represent mean MIC_{90}.

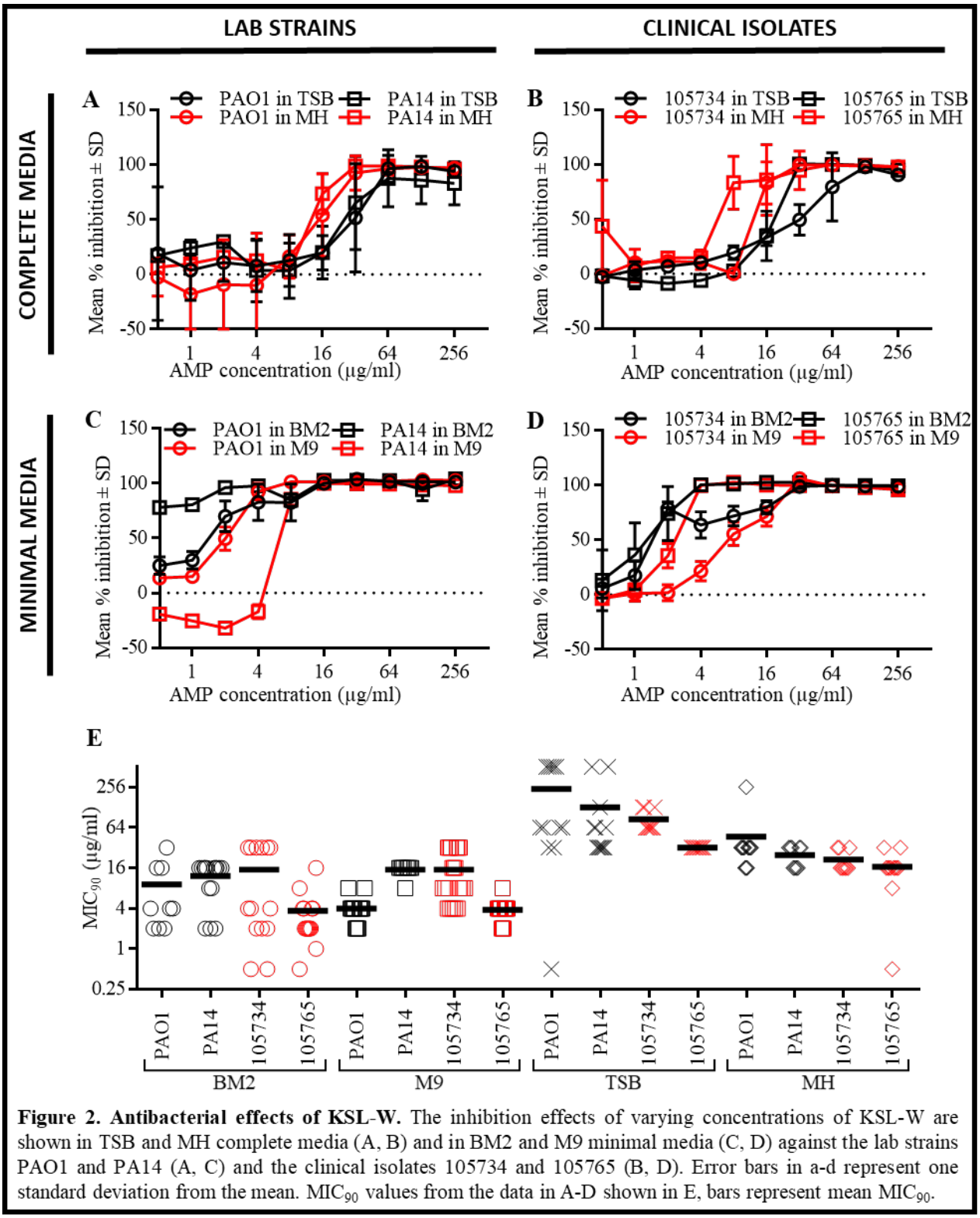

Figure 2. Antibacterial effects of KSL-W. The inhibition effects of varying concentrations of KSL-W are shown in TSB and MH complete media (A, B) and in BM2 and M9 minimal media (C, D) against the lab strains PAO1 and PA14 (A, C) and the clinical isolates 105734 and 105765 (B, D). Error bars in a-d represent one standard deviation from the mean. MIC_{90} values from the data in A-D shown in E, bars represent mean MIC_{90}.

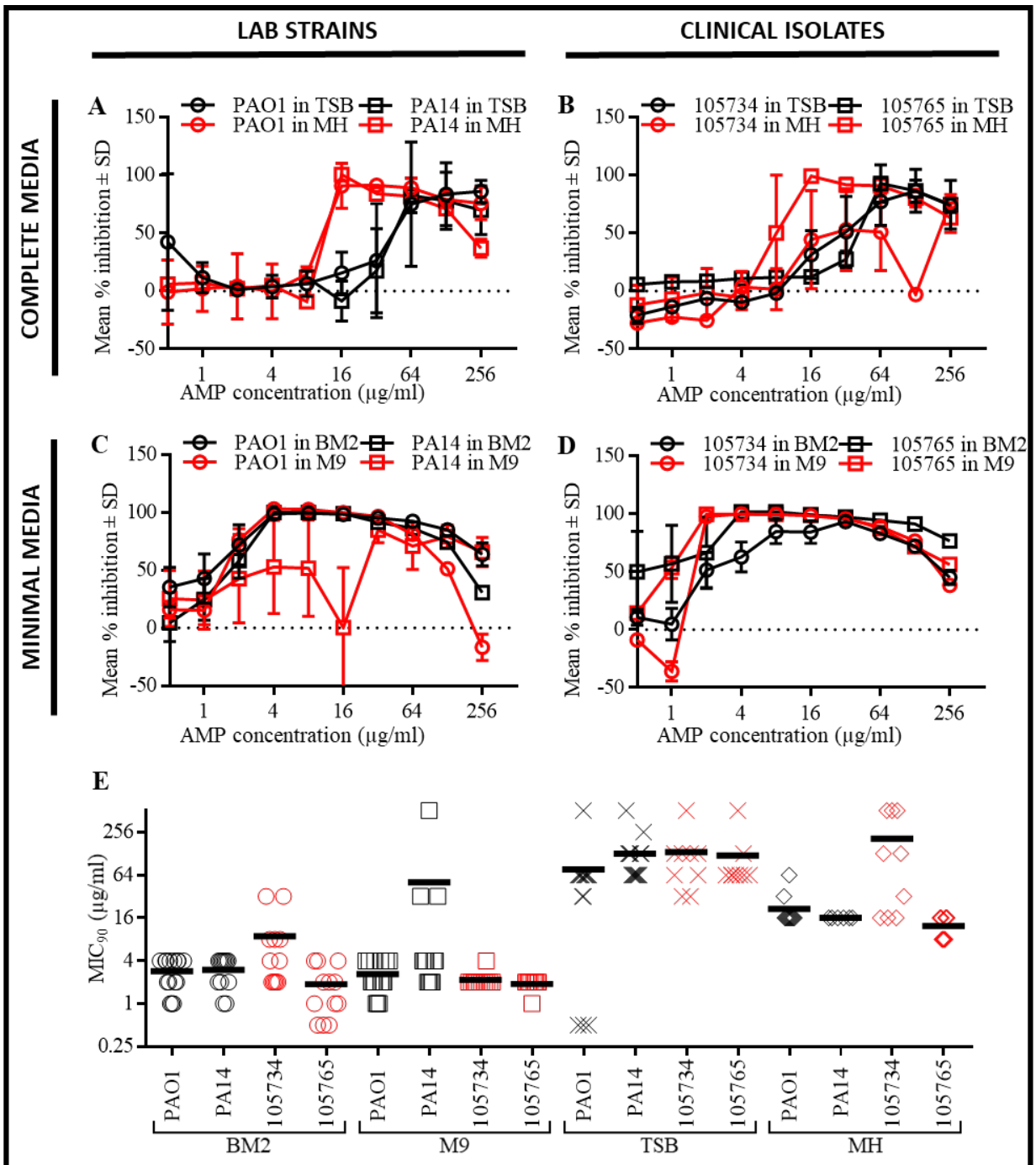

Figure 3. Antibacterial effects of K_6L_9. The inhibition effects of varying concentrations of K_6L_9 are shown in TSB and MH complete media (A, B) and in BM2 and M9 minimal media (C, D) against the lab strains PAO1 and PA14 (A, C) and the clinical isolates 105734 and 105765 (B, D). Error bars in A-D represent one standard deviation from the mean. MIC_{90} values from the data in A-D shown in E, bars represent mean MIC_{90}.

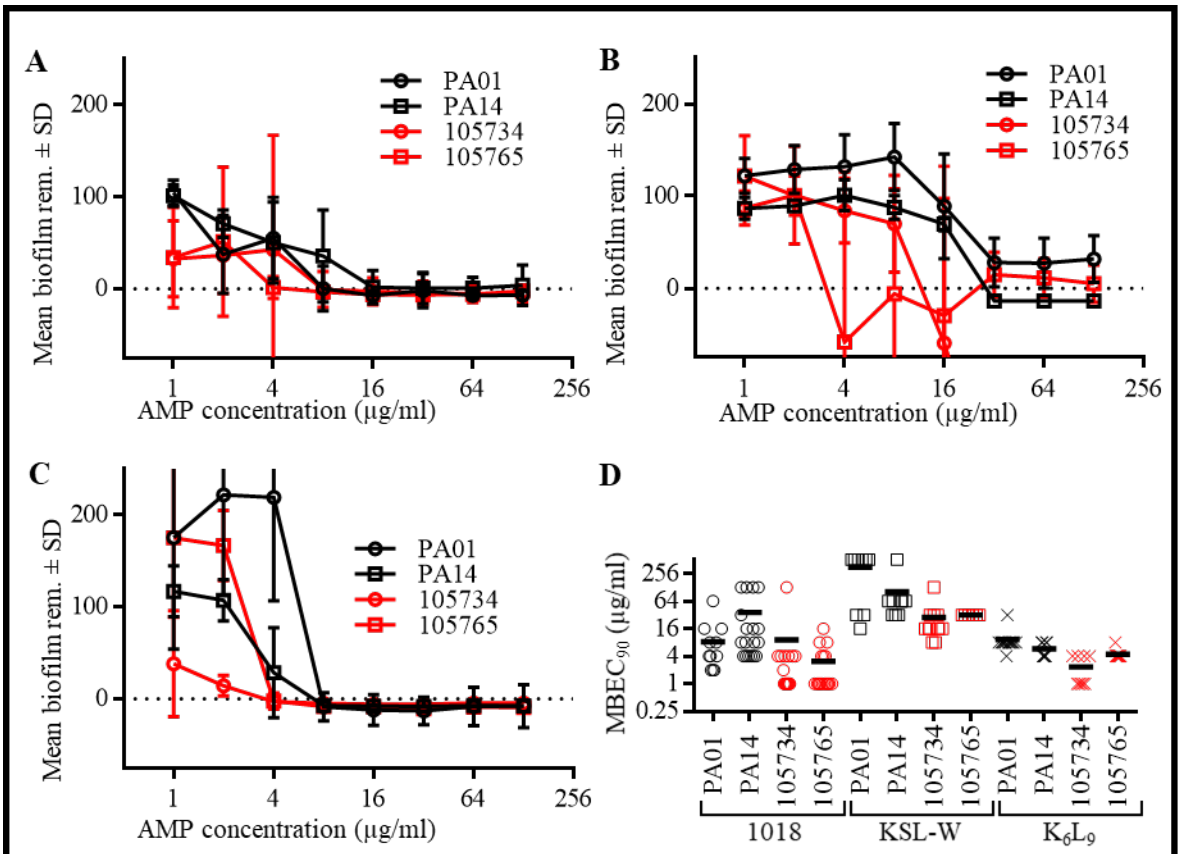

Figure 4. Antibiofilm effects of 1018, KSL-W, and K_6L_9. The mean percent biofilm remaining, determined by crystal violet staining, of 24-hour-old biofilms after incubation with varying concentrations of 1018 (A), KSL-W (B), or K_6L_9 (C) for 6 hours. Error bars in A-C represent one standard deviation from the mean. $MBEC_{90}$ values from the data in A-C shown in D, bars represent mean $MBEC_{90}$.

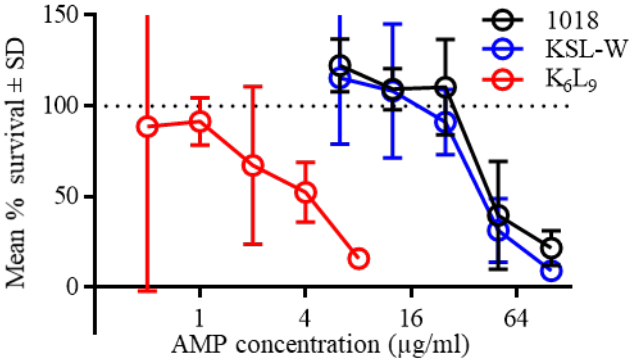

Figure 5. Cytotoxicity of 1018, KSL-W, and K_6L_9 to human gingival epithelial cells. The mean percent cells surviving after 24-hour incubation with varying concentrations of 1018, KSL-W, or K_6L_9 measured by the cell proliferation reagent (WST-1). Error bars represent one standard deviation from the mean.

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14. ABSTRACT

Multidrug resistant (MDR) bacteria, and nosocomial infections are increasingly common, with military personnel and the general population. However, few novel antibiotics are currently in development to combat them. The use of antimicrobial peptides (AMPs) is one new avenue currently under development to address the global need for new antibacterial therapies. On the other hand, as experiments use varying growth conditions, target species or strains, and doses, research consensus on AMP efficacy is limited. The purpose of this study was to evaluate the effect of bacterial growth conditions on AMP antibacterial activity, and set a reasonable standard for objective comparison between AMPs. Three AMPs were selected and tested against Pseudomonas aeruginosa, an opportunistic pathogen of international concern. AMP selection was based on their peptide size, mode of action, efficacy, and cytotoxicity. This evaluation was designed to balance antibacterial and antibiofilm activity against cytotoxicity levels. Standardized antibacterial activity assays were conducted with three selected AMPs against four varieties of P. aeruginosa; two laboratory strains and two MDR clinical isolates. Bacteria were exposed to various growth conditions; two different minimal and two different complete media. AMPs were also tested for antibiofilm activity and cytotoxicity in human gingival epithelium progenitor cells. Antibacterial and antibiofilm efficacies varied by over 100-fold with target bacterial strain and growth conditions. AMP 1018 disrupted biofilms at low concentrations while K6L9 was effective against both laboratory strains and biofilms. KSL-W showed generally better antibacterial activity but was less effective in disrupting biofilms than 1018. Culture medium had a larger impact on AMP efficacy than strain identity, including MDR status. Changes in bacterial growth conditions have large effects on AMP efficacy. Standardized protocols and growth conditions can minimize these effects and enable reasonable comparison of AMP antibacterial or antibiofilm effects across AMPs and between research groups.

15 . SUBJECT TERMS
multidrug resistant bacteria, antimicrobial peptide, Pseudomonas aeruginosa, antibiofilm

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