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Underscoring inter-strain variability and the impact of growth conditions on associated antimicrobial susceptibilities in pre-clinical testing of novel antimicrobial drugs

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Abstract

In the era of multi-drug resistant (MDR) organisms, reliable efficacy testing of novel antimicrobials during developmental stages is of paramount concern prior to introduction in clinical trials. Unfortunately, inter-strain variability is often underappreciated when appraising the efficacy of innovative antimicrobials as pre-clinical testing of a limited number of standardized strains in unvarying conditions does not account for the vastness and potential for hyperdiversity among and within microbial populations. In this review, the importance of accounting for inter-strain variability's potential to impact breadth of novel drug efficacy evaluation in the early stages of drug development will be discussed. Additionally, testing under varying microenvironmental conditions that may influence drug efficacy will be discussed. Biofilm growth, the influence of polymicrobial growth, mechanisms of antimicrobial resistance, pH, anaerobic conditions, and other virulence factors are some of critical issues that require more attention and standardization during pre-clinical drug efficacy evaluation. Furthermore, potential solutions for addressing this issue in pre-clinical antimicrobial development are proposed via centralization of microbial characterization and drug target databases, testing of a large number of clinical strains, inclusion of mutator strains in testing and the use of growth parameter mathematical models for testing.

Keywords

antimicrobials; infection; resistance; strains; variability

INTRODUCTION

The advent of antimicrobials played a critical role in the 20th century's precipitous decline of infectious disease mortality (Giske et al. 2008; Aminov 2016). However, antimicrobial resistance has stymied our progress against infectious diseases as morbidity and mortality

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are on the rise (Cosgrove 2006). The ubiquity of drug resistant organisms has spawned a multipronged effort to circumvent nosocomial and community emergence of these notoriously difficult to treat pathogens (Thabit et al. 2015). Although prudence of antibiotic use and reduction of resistant organism spread by implementing effective disinfection methods are essential efforts to address this public health epidemic, the development of innovative antimicrobials are crucial for changing the course towards a post-antibiotic era (Draenert et al. 2015).

Unfortunately, strain-to-strain variance is underappreciated when appraising novel antimicrobial efficacy at the pre-clinical stage. Investigators often utilize a limited amount of ATCC standard strains or few clinically acquired strains when testing efficacy of new drug delivery vehicles, peptides, compounds, derivatives, etc. Testing one or a few pathogenic standardized genetic variants does not constitute a thorough understanding of drug efficacy due to a lack of uniformity to account for mutations that lead to resistance across pathogenic microbial populations as well as the plethora of environmental conditions that impact drug responses. Via the inclusion of multiple strains in pre-clinical studies, investigators may be able to preemptively identify potential mechanisms of resistance the drug of interest is predisposed to based on drug failure against microbial strains with well-characterized resistance mechanisms. Early stage identification of resistance mechanisms that interfere with drug efficacy can inform enhanced iterations of the antimicrobial of interest, as well as future drug design for antimicrobials with similar chemical properties or mechanism of action.

Given that the biggest hindrance to the advancing of experimental drug development at later stages, i.e. phase III clinical trials, is inadequate efficacy, earlier studies that attempt to manipulate physiological factors that may be contributing to unexplained drug failure can help detect pitfalls earlier rather than later in the timeline of development. Indeed, among 640 novel therapeutics tested in phase III clinical trials from 1998 to 2008, 344 failed in clinical development and 57% of those drugs were shown to have inadequate efficacy after rigorous testing in animal models that proved a level of efficacy deemed sufficient for progress to clinical trials (Hwang et al. 2016). Some of the unanticipated treatment failures that occur in certain physiological milieus may be circumvented by testing antimicrobial efficacy in varying *in vitro* environmental conditions during early pre-clinical studies (Table 1). Prominent environmental conditions that fluctuate depending on the anatomical setting are nutrient access, pH, and relative oxygenation (Table 1). Nutrient limitation often leads to biofilm formation, a clinically salient survival phenotype that drastically affects antimicrobial efficacy for many commonly used agents. pH deviations can impact the chemical structure of different classes of encouraging antimicrobials, namely animal-derived peptides (Table 1). Oxygen-rich versus oxygen-depleted microenvironments can also impinge upon an antimicrobial's ability to fully eradicate targeted pathogens as has been shown with a slew of commonly used antibiotics that are effective in the proximal GI track with subsequent loss of activity upon reaching the distal intestine (DeMars et al. 2016). Pre-clinical compound testing issues also can be found at earlier stages--databases detailing a pathogen's wealth of disease mechanisms and hypothetical drug targets offer an overwhelming amount of data that is haphazard and not integrated for ease of drug identification prior to initiation of pre-clinical testing (Ekins, Clark, et al. 2014).

The aim of this mini-review is to outline the reasons why underscoring microbial diversity when assessing drug efficacy during early drug development stages is of paramount concern in the context of antimicrobial resistance and drug failure in clinical trials. Centralizing and unification of microbial characterization and drug target databases may help to expedite promising compounds in the drug design pipeline while underscoring the role that microbial diversity bears on drug efficacy (Table 2). Inclusion and standardization of a certain threshold of clinical strains in conjunction with hypermutator phenotypes with mutation rates up to 10^4 compared to wild-type strains will be explored as potential solutions to enhance pre-clinical testing of novel candidate antimicrobials (Table 2). Furthermore, taking a cue from the food and agriculture industry, standardizing testing strategies for vetting antimicrobial efficacy across extensive environmental parameters and strains will also be discussed to help pinpoint flaws in drug development at the pre-clinical or basic science stages (Table 2). Ultimately, these proposals may help to curb mortality caused by multi-drug resistant (MDR) infectious agents estimated to be ~23,000 per year in the United States (US) as well as the resulting hospital-associated economic burden of MDR infections reaching as high as \$18,588 to \$29,069 per hospitalized patient (Roberts et al. 2009; Michaelidis et al. 2016).

The diversity of inter-strain biofilm phenotypes and resulting antimicrobial susceptibility

Biofilms are aggregate microbial communities encased in an extracellular polymeric matrix that often showcase extreme tolerance to our current antimicrobial arsenal (Bjarnsholt 2013). This highly conserved phenotype has also been implicated in the pathogenesis of chronic infections (Bjarnsholt 2013). Tolerance of antibiotics is not to be confused with antibiotic resistance given that many pathogens become susceptible to certain antibiotics upon biofilm disruption (Bjarnsholt 2013). Inability of antibiotic penetration based on biofilm mechanical barrier properties is thought to play a prominent role of antibiotic tolerance among certain strains. Some other proposed tolerance mechanisms hinged upon architectural blockade include biofilm multilayer structure, the presence of an extracellular slimy matrix and antibiotic adsorption by the microbial cells (Singh et al. 2016). Conversely, diffusion limitation based on ionic interactions or deployment of antibiotic degrading enzymes outline biomolecular properties inherent to biofilm phenotypes that may also contribute to antimicrobial tolerance (Singh et al. 2016).

Strains from the same species showcasing biofilm phenotypes may have heterogeneous susceptibilities to antimicrobials and virulence. Indeed, on chloramphenicol treated staphylococci spp. and *Klebsiella pneumoniae* biofilms, there was strain dependent impediment of antimicrobial action (Singh et al. 2016). Biofilm permeating capacity was also strain dependent for *K. pneumoniae* clinical isolates treated with amikacin and the ATCC strains of *Staphylococcus aureus* treated with cefotaxime that was most likely explained by dissimilar penetration (Singh et al. 2016). *S. aureus* strains, for example, may utilize cell wall bound autolysin with fibronectin-binding proteins or the *ica-ADBC* gene cluster dependent polysaccharide intercellular adhesin (PIA) and the polymeric *N*-acetylglucosamine for biofilm formation. Indeed, deletion of the major autolysin gene *atl* has been shown to impair fibronectin-binding protein biofilm production on hydrophilic polystyrene in 12 clinical methicillin-resistant *S. aureus* (MRSA) isolates but had no effect

on PIA-dependent biofilm production by 9 methicillin-susceptible *S. aureus* (MSSA) isolates (Houston et al. 2011). Fibronectin-binding protein dependent biofilms were also noted to be more susceptible to antibodies against penicillin binding proteins when compared to antibiotics (Beceiro et al. 2013).

Of particular interest, *Pseudomonas aeruginosa* has a prominent role as a biofilm-forming organism ubiquitously implicated in chronic respiratory infections for cystic fibrosis patients (Doring et al. 2011). Cystic fibrosis (CF) is an autosomal recessive disease characterized by ineffective transport of chloride and sodium ions across a mutated transmembrane channel in epithelia that disproportionality affects Caucasian males with a carrier frequency of 1:25. As alluded to previously, the clinical manifestations of CF entail chronic pulmonary infections due to the lack of water diffusion across tissues leading to viscous mucus accumulation, a niche which promotes biofilm growth due to extensive tissue destruction and consequential remodeling as a result of immunological overstimulation (Doring et al. 2011). *P. aeruginosa* presents a rather difficult clinical conundrum given the microbe's extensive list of virulence factors and uncanny ability to resist nearly every disinfectant in our arsenal while persisting in a milieu that precludes adequate drug infiltration for proper eradication (Hancock 1998). Even though *P. aeruginosa*'s resistance is often attributed to its decreased outer membrane permeability and to antibiotic efflux apparatuses, genomic sequencing has underscored the relevance of the organism's large library of up to 521 regulatory genes out of a 6.3 Mb genome (Stover et al. 2000; Doring et al. 2011). In the context of biofilms, one of the regulatory genes playing an interesting role is NfxB whose inactivation leads to upregulation of MexCD-OprJ efflux pump. Upregulation of MexCD-OprJ unintuitively decreases inducible AmpC β -lactamase and other efflux pumps, resulting in what can be easily misinterpreted as deleterious cell envelope changes in the setting of β -lactam antibiotic assault. However, decrease in the periplasmic β -lactamase activity via this mechanism apparently produced by an abnormal permeation of AmpC out of the cell into the biofilm matrix resulting in enhanced resistance at the communal level rather than the single cell level (Mulet et al. 2011). Thus, these regulatory mechanisms may favor biofilm phenotypes found in CF patients. Moreover, a variety of morphotypes not typically encountered in environmental *P. aeruginosa* strains have been described including small colony variants (SCVs) that often lead to increased persistence in damaged airways within this patient population (Haussler et al. 1999). SCVs display superior immune evasion via tolerance to reactive oxygen species (ROS) compounds, HOCl and H₂O₂, and production of virulence factors such as type III secretion system components and siderophore production. Collectively, these data may explain SCV's significant correlation with prolonged antibiotic use and parameters revealing poor lung function (Haussler et al. 1999; Pestrak et al. 2018). The SCV phenotype is not exclusive to *P. aeruginosa* strains and is known for prolific biofilm growth across many species including *S. aureus*, an organism that is the most commonly isolated pulmonary pathogen in CF patients during the first decade of life (Keitsch et al. 2018). In fact, factors contributing to establishment of tenacious biofilms are expressed synergistically during co-infection of *P. aeruginosa* and *S. aureus*; denser biofilms, serine-rich adhesions, and extracellular matrix binding proteins were found to be culpable in aminoglycoside resistance and a sturdier co-habitation biofilm structure (Kumar and Ting 2015).

Single species biofilm heterogeneity is not limited to bacterial pathogens as clinical and laboratory strains of *Candida albicans* have also demonstrated varying capacity to create biofilms (Alnuaimi et al. 2013). *C. albicans* biofilms have significant implications in hospital-acquired infections given that it is the most common fungal pathogen isolated from medical devices such as urinary and central venous catheters, pacemakers, mechanical heart valves, joint prostheses, contact lenses, and dentures; not surprisingly, this commensal pathogen ranks fourth for microbial agent isolated from bloodstream infections (Henriques et al. 2006; Dominic et al. 2007; Nobile and Johnson 2015). *Candida* biofilms are largely considered to be more resistant to our current antifungal armamentarium making characterization of fungal biofilm susceptibility across varying biofilm forming strains imperative (Nobile and Johnson 2015). Previous studies have defined specific low biofilm forming versus high biofilm forming capabilities across unique *C. albicans* strains (Alnuaimi et al. 2013; Sherry et al. 2017). This heterogeneity has relevance in pathogenicity as subgingival *C. albicans* genotype A strains from diabetic patients were shown to have increased cell surface hydrophobicity indicating an inability to form deeper tissue infections in comparison to genotypes B and C (Alnuaimi et al. 2013). Indeed, *C. albicans* clinical strains undergo genomic changes at a similar rate to cancer cells, especially during laboratory passaging; this high level of genomic plasticity highlights the need to underscore diversity of strains in the context of biofilm formation when evaluating drug efficacy (Selmecki et al. 2010).

Polymicrobial infections in the setting of biofilm phenotypes highlight the relevance of characterizing interactions among certain species and strains in the context of drug development. Biofilms that are polymicrobial in nature pose a conundrum for eradication of infection as the coexistence of two or more microbial species in a static three-dimensional milieu can lead to interactions that determine drug susceptibility (O'Connell et al. 2006). Also, polymicrobial infections have been shown to intrinsically alter the dynamics of disease severity and course as a result of these synergistic intercellular interactions (Liao et al. 2014). A well characterized phenomenon occurring in mixed infections is indirect pathogenicity (IP), a circumstance where drug-resistant organisms that are deemed to have low virulence secrete factors that shield antibiotic-sensitive pathogens from elimination (Fung et al. 1994; O'Connell et al. 2006; Liao et al. 2014). The occurrence of IP in microbial biofilms have broad significance given that biofilm phenotypes are implicated in an array of hard to treat infectious processes including ventilator-associated pneumonia, wounds, and abscesses (O'Connell et al. 2006). A prime example is *Acinetobacter baumannii*, an organism that has been shown to confer protection for carbapenem-susceptible organisms and has been stressed as an organism of "serious threat" by the Center for Disease Control and Prevention due to 68% of infections demonstrating multidrug resistance including resistance against polymyxin, a last resort antibiotic (Solomon and Oliver 2014; Girardello et al. 2017). Coculturing of carbapenem-resistant *A. baumannii* and carbapenem-susceptible *Escherichia coli* strains has indicated that *E. coli* is safeguarded in the presence of imipenem because of extracellularly secreted carbapenemases often isolated from biofilm matrixes (Hoiby et al. 2010; Liao et al. 2014). Previous studies suggested that extracellular β -lactamases were released as a consequence of cell lysis after being subjected to antimicrobials, but recent work rejects this notion due to the absence of recoverable gyrase

(Liao et al. 2014). β -lactamase protection was also noted in interactions between β -lactamase sensitive pneumococci and β -lactamase positive *Moraxella* (Budhani and Struthers 1998). *M. catarrhalis* has clinical relevance as the third most commonly isolated pathogen from respiratory tract secretions and is implicated in acute otitis media, chronic obstructive pulmonary disease, and acute rhinosinusitis (Fung et al. 1994; Budhani and Struthers 1998). In polymicrobial infections, at least 90% of *M. catarrhalis* isolates produce a β -lactamase with activity against penicillin and the amino penicillins that have been shown to confer protection for otherwise susceptible organisms. *Streptococcus pneumoniae* was protected at antibiotic concentrations indicating resistance when concomitantly cultured with lactamase-producing *Moraxella* in media containing benzylpenicillin or amoxicillin even though β -lactamase levels in biofilm were significantly lower than those in broth culture supernatants (Fung et al. 1994). Therefore, in this instance, the inherent physicality of biofilm structures may very well work in tandem with biochemical weaponry to create resistance against antimicrobials. Periodontal disease, an infection with global distribution affecting 537 million individuals, also epitomizes the intimate connection between mixed infections and biofilm formation in particular anatomical niches (Disease et al. 2016). Periodontal disease is the result of chronic, polymicrobial gram-negative bacterial biofilm accumulation in the gingival margin causing loss of dental attachment, reduction in bone level, and can culminate in tooth loss if left untreated (Lee et al. 2012). Considering the backdrop of a microbe rich environment containing up to 500 different bacterial species, investigators are now approaching the testing of new modalities like photosensitizer therapy while strongly considering the likelihood that multiple pathogens like *Actinomyces naeslundii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* will be inhabiting subgingival pockets simultaneously (Cieplik et al. 2018). Furthermore, classically MDR organisms have been recovered from periodontal biofilms, including *Enterococcus faecalis* in up to 51.8% of periodontitis patients (Balaei-Gajan et al. 2010). These MDR microbes may influence systemic antimicrobial therapy for severe periodontal disease and have the capacity to contribute to disease progression in severe subgingival infections that are clearly polymicrobial in nature (Bhardwaj et al. 2017; Feres et al. 2018). Thus, when testing new modalities against biofilm producing organisms, researchers should avoid testing drugs in systems that are devoid of other commensals that have the ability to interfere with drug activity and must also consider the heterogeneity of biofilm formation that undoubtedly impacts evaluation of an innovative drug's efficacy.

Wide spectrum of antimicrobial resistance seen across bacterial species necessitates testing of multiple strains

Overlooking strain heterogeneity may preclude investigators' capacity to reliably interpret mechanisms of microbial adaptation to our biochemical weaponry. As previously mentioned, *A. baumannii* has serious clinical consequences, namely due to its prominence as a MDR organism with a multiplicity of putative mechanisms of resistance (Solomon and Oliver 2014; Girardello et al. 2017). Some well described mechanisms entail membrane *pmrA*/*pmrB*-mediated lipopolysaccharide (LPS) modification via addition of phosphoethanolamine in the lipid A moiety and efflux pumps with the capacity to excrete numerous drugs (Beceiro et al. 2011; Henry et al. 2015). Another resistance mechanism involves loss of LPS due to a single nucleotide mutation on *lpxA*, *lpxC*, or *lpxD*, operon constituents required for LPS

biosynthesis (Girardello et al. 2017). Troublingly, in the presence of polymyxin, additive mechanisms of resistance can be inconsistently induced in certain strains of *A. baumannii* that have already developed one of these aforementioned polymyxin resistance mechanisms. Strains with established resistance had drastically increased minimum inhibitory concentrations (MIC; 0.25 µg/mL to >64µg/mL) upon re-exposure to polymyxin that was variable across strains (Girardello et al. 2017). Alarming, the *A. baumannii* strain LAC-4, a clinical specimen collected from a Los Angeles County hospital nosocomial outbreak, has been labeled as hypervirulent based on reproducibility of human pulmonary infection in a mouse model causing 100% mortality post intranasal inoculation (Ou et al. 2015). Comparative genomic studies with 19 other strains acquired from the same clinical setting demonstrated that LAC-4 harbors 12 and 14 copies of unique insertion sequences and three novel composite transposons which confer resistance genes. Furthermore, newly identified genomic islands were also found to carry genetic determinants for LAC-4's K capsule phenotype, a virulence factor of major interest (Russo et al. 2010; Ou et al. 2015). This is of particular significance given that most clinical isolates and laboratory strains used in mouse models fail to adequately replicate disease in immunocompetent hosts characterized by pneumonia that results in extensive hard-to-treat extrapulmonary dissemination; previous studies relied on the use of neutropenic C57BL/6 mice in order to resemble human disease processes attributed to *A. baumannii* (van Faassen et al. 2007; Ou et al. 2015). Descriptions of these phenomena are of interest to investigators because novel antimicrobials must be able to circumvent all potential permutations of defined resistance mechanisms and must be tested in animal models that truly replicate disease processes in the human host. To best capture a broad breadth of these variations, a wide sampling of phenotypes in the appropriate settings must be tested when assessing efficacy of new drugs.

The rationale for testing several strains is further bolstered by evidence describing large-scale community outbreaks of MDR organisms not previously exposed to certain antimicrobials. Notably, there have been nursing home outbreaks of ceftazidime-resistance encoded amongst multiple plasmids in several *E. coli* and *K. pneumoniae* strains without a documented site of nosocomial origin (Wiener et al. 1999). This is in sharp contrast to previous long-term care facility MDR outbreaks demonstrating up to 53% of infected patients had prior antibiotic exposure (Rice et al. 1990; Wiener et al. 1999). Isolates from 20 nursing home patients having a positive culture upon hospital admission revealed 7 different types of resistant strains harboring 4 unique plasmids that conferred β-lactamase ceftazidime resistance. Moreover, three different strains were isolated from three patients found sharing the same living quarters, also highlighting the importance of effective disinfection in healthcare facilities. These data lend credence to the importance of recognizing insurmountable strain heterogeneity within one microbial ecosystem, community and hospital setting alike, as molecular mechanisms of resistance may not be shared uniformly across strains.

Of note, there are numerous studies highlighting individual patients infected with more than one isolate of the same organism demonstrating inconsistent susceptibilities to antimicrobials. This phenomenon, known as heteroresistance, may also be considered a transient, preliminary stage preceding full resistance (Folkvardsen et al. 2013). Heteroresistance has been described in case reports of *Mycobacterium tuberculosis*, *S.*

aureus, and *Helicobacter pylori* infections. Heteroresistant infection studies with *H. pylori* have also concluded that these isolates were the product of high genomic diversity that evolved over a long period of time rather than concomitant infection with two completely unique strains (Kao et al. 2014). Mechanisms of heteroresistance have been well characterized in methicillin-resistant *S. aureus* as these *mecA* positive organisms have the capacity to create low-affinity penicillin binding proteins that confer β -lactam resistance. Diagnostic implications for heteroresistance have been emphasized given studies that have shown inadequate identification of the previously mentioned pathogens. For example, greater than 1% of rifampin-resistant *M. tuberculosis* strains (in artificially recreated heteroresistant strain pools) were not identified using traditional culturing, probe assays, and sequencing (Folkvardsen et al. 2013).

Naturally, mechanisms of persistence and adaption to thwart host defenses and antimicrobials vary across strains. Often the acquisition of antibiotic resistance occurs with a cost to fitness that results in being outcompeted by wild-type organisms in a setting lacking antibiotics (Olivares Pacheco et al. 2017). For instance, *S. aureus* strains acquiring oxacillin resistance present with attenuated virulence. In *A. baumannii*, modulation of virulence also occurs upon gaining of resistance given that polymyxin susceptible clinical strains exhibited reduced fitness and virulence upon exposure to the antibiotic. Similarly, *A. baumannii* ATCC 19606 strain also showcased impaired fitness and virulence upon exposure to colistin, another antimicrobial of last resort (Wiener et al. 1999; Andersson and Hughes 2010; Beceiro et al. 2013). Yet, colistin resistance was observed in strains with prolonged drug exposure which may also potentiate fitness and virulence leading to increased severity of disease. Collectively, these examples offer a glimpse into the diversity of ways strain variability impacts drug resistance, and consequently, virulence and fitness. Therefore, those strains lacking conferred antimicrobial resistance may possess increased virulence that should not be discounted when assessing novel antimicrobials.

Robust characterization of inter-strain diversity via modification of growth parameters including nutrient limitation, pH, salt concentration, and anaerobic conditions

To a large degree, microbial phenotype diversity, including drug resistant phenotypes, is influenced by exogenous environmental pressures (O'Neill and Chopra 2001). In a similar vein, the food and agricultural industry is cognizant of how microbial strain diversity plays a profound role in spoilage of goods. Putrefaction of crops and escalating concerns of product safety are motivating factors to optimize the reduction of pathogens and spoilage microorganisms via risk assessment studies (den Besten et al. 2017). These studies of food-borne infectious agents are often predicated on robust characterization of numerous strains of culprit pathogens to reliably predict patterns of contamination and subsequent infection. These study frameworks may be effectively implemented in the pre-clinical drug development setting to prognosticate patterns of antimicrobial resistance across species (O'Neill and Chopra 2001). For instance, amongst 20 laboratory and environmentally acquired strains of food-borne pathogens *Listeria monocytogenes* and *Lactobacillus plantarum*, inactivation and growth characteristics were noted to be significantly impacted by inter-strain variation when using mathematical models to determine cardinal growth parameters. Inter-strain diversity was noted as a moderate impact variability factor

influencing growth when appraising temperature minimum, pH minimum, water activity growth limit, and maximum unassociated lactic acid concentration (den Besten et al. 2017). Fluctuation in D-values, or decimal reduction dose required for eradication of 1 log of the exposed pathogens in a distinctive setting, was highly impacted when considering inter-strain variation across both species. In another study assessing intra-species variation and its effect on growth kinetic behavior across 60 isolates of *Salmonella enterica*, the variability of maximum specific growth rate (μ_{max}) among the *S. enterica* strains was greater than that observed within strain replicates. Moreover, pH and saline rich conditions provoked increased variation as the μ_{max} coefficient of variation among the tested strains at pH 7.0–0.5% NaCl was 6.1%, while at pH 4.3–0.5% NaCl and pH 7.0–6.0% NaCl was 11.8% and 23.5%, respectively (Lianou and Koutsoumanis 2011). This exhaustive characterization of strains via modulation of environmental factors can enhance efficacy and/or resistance potential assessments for early stage antimicrobial development and characterization.

In the setting of disease, these shifts in microbial fitness across strains are not inconsequential. Safeguards against acidic conditions have been well-characterized in pathogens affecting the gastrointestinal tract (Williams 2001). For instance, in patients receiving enteral nutrition via percutaneous endoscopic gastrostomy (PEG) for management of severe dysphagia, pathogenic strains of organisms that are not typically found in the caustic environment of the stomach (0–2 pH) can be isolated in gastric aspirates (O'May et al. 2005). *C. albicans*, *E. coli*, and *Streptococcus spp.* were found to supplant typical microbiota residents of the stomach such as *H. pylori* and lactobacilli (Williams 2001). These adaptations of acidic resistance in organisms originally sensitive to these conditions (likely migrants from the non-acidic duodenum) are the result of genetic selection via acquisition of an acid tolerance response that may not be seen across all strains (Williams 2001; O'May et al. 2005). Tolerance to high salt environment or halotolerance across strains also bestows advantages to microbial survival. Recently uncovered Na^+ mediated antimicrobial defenses and enhancement of macrophage activation in the skin may be overcome by pathogenic strains possessing halotolerance (Jantsch et al. 2015). Strains of *E. coli* that cause urinary tract infections also exhibit the ability to thrive in a hypertonic milieu. For example, 301 *E. coli* clinical isolates from blood, urine, stool and 12 enteric strains had varying tolerance to NaCl in minimal media (Kunin et al. 1992). *In vitro* growth tolerance to secondary bile acids like lithocholic acid has also been documented in *Clostridium difficile* across 33 strains with a correlation to severity of pseudomembranous colitis in patients from which the strains were isolated (Lewis et al. 2017). *C. difficile* strain growth response in bile acid was noted to be highly variable across this impressive number of tested strains; notably, new evidence has surfaced suggesting that treatment resistance may be due to the coexistence of residential *Clostridium spp.* in the intestinal microbiome that are able to effectively dehydroxylate bile acids (Buffie et al. 2015). This capacity for *C. difficile* growth in bile rich environments may be impacting vulnerability to current antimicrobial arsenal which addresses the importance of testing new drugs while emulating the vast array of native microenvironments a pathogen may encounter during colonization and subsequent disease state.

These strain variable microbial adaptations can also affect the efficacy of a newer generation of antimicrobial modalities. For one, the problem of salt sensitivity for antimicrobial

peptides has hindered their development as a possible anti-infective treatment (Yu et al. 2011). Salt-rich environments such as bronchopulmonary fluids in patients with CF inactivate β -defensin. Clinically active histidine-rich peptides such as clavanins also exhibited similar efficacy interference (Yu et al. 2011). Also, N-chloramines are compounds that are candidate drugs being explored for their bactericidal properties, however, these compounds are pH sensitive and microbes harboring mechanisms to withstand acid-base changes could easily escape the drug's effects (Gottardi et al. 2013). Similarly, organisms with salt tolerance would likely be able to evade the effects of peptide antimicrobials. For one, inactivation of the *putP* homologue, an osmoprotective high-affinity proline permease, in *S. aureus* significantly reduces virulence in an experimental endocarditis model (Bayer et al. 1999). Likewise, the food-borne pathogen *L. monocytogenes* thrives in 0.3 to 0.7 M NaCl by using the tissue abundant carnitine as an osmolyte and has also been shown to use OpuC-encoded osmolyte uptake for proliferation and survival in mouse models (Sleator et al. 2001). Studies with halotolerant organisms demand extensive testing for experimental drugs at preliminary stages since many organisms possess the capacity to survive extreme salt conditions that other organisms would undoubtedly succumb to.

Facultative bacteria pose a problem for evaluating the efficacy of new drugs given that certain microbicides elicit diverse responses contingent on the presence or absence of oxygen. This is illustrated by a glaring issue in protocols evaluating the consequences of routinely administered antimicrobials on livestock wellbeing, principally cattle: perturbation of facultative and anaerobic residential bacteria found in cattle is assessed by using pharmacodynamic models predicated on MIC assays conducted in aerobic conditions (DeMars et al. 2016). These models neglect the impact anaerobic conditions have on antimicrobial efficacy in the distal intestine as studies have demonstrated that ampicillin, ceftriaxone, gentamicin, kanamycin, erythromycin, azithromycin, sulfamethoxazole/trimethoprim and tetracycline all had contrasting effects on growth across multiple strains for facultative organisms in anoxic conditions (DeMars et al. 2016). Corroborating this notion, antistaphylococcal activities of five fluoroquinolones under both aerobic and anaerobic conditions were also tested showing that although all five agents were bactericidal under both aerobic and anaerobic conditions, the bactericidal activity of all agents was delayed by anaerobiosis (Zabinski et al. 1995). A slew of external factors that may have impinged on fluoroquinolone efficacy in anaerobic conditions were assessed including hydrophobicity, intracellular pH, antibiotic concentration, and structure-activity relationships but none of mentioned parameters were found to have influenced differences in activity (Zabinski et al. 1995). Intersecting with previously discussed biofilm disease processes, *P. aeruginosa* mucoid biofilms can thrive anaerobically with the help of two formerly unidentified proteins, OprF and rhl, an outer membrane protein and quorum sensing circuit protein, respectively (Hassett et al. 2002). Knocking out of these gene products results in inadequate biofilm growth because of shutting off of nitrite reductase and stimulation of metabolic suicide via overproduction of nitric oxide. These *P. aeruginosa* biofilm virulence factors are contributory for ineffective and attenuated bactericidal activity of many frontline antimicrobials in anaerobic circumstances (Hassett et al. 2002; Hill et al. 2005). Collectively, these studies stress the importance of preclinical drug testing against facultative pathogens must include oxygen-rich and oxygen-depleted scenarios for thorough understanding of

efficacy. A potential recommendation demands thorough antimicrobial testing for clinically acquired bacterial species that are notorious for biofilm formation as aerobic planktonic cells and biofilms, and anaerobic planktonic cells and biofilms. By standardizing these procedures, we may help guide the development of antimicrobials that perform optimally in a given microenvironment for a specified disease process rather than for the implicated pathogen (i.e., *P. aeruginosa* existing as a persistent biofilm in anaerobic conditions for CF patients).

Testing of α -defensins for activity against facultative intestinal microbes may attest to the impact of anaerobic conditions on the appraisal of a therapeutics' potency against infection, with special consideration for antimicrobial peptides. Crp2, Crp3 and Crp4 are mouse-derived enteric Paneth cell α -defensins, termed cryptins, that were tested for their capacity as antimicrobials given previous data showing Crp4's selective bactericidal activities against certain, but not all, anaerobic bacterial species (Mastroianni et al. 2014). In the presence of oxygen over titrated concentrations of peptide Crp4 demonstrated bactericidal activities against *Salmonella typhimurium* and *Shigella flexneri*. Conversely, in conditions deprived of oxygen, Crp4 bactericidal activity was significantly decreased against both *S. flexneri* and *S. typhimurium*, in varying degrees relative to the organism (Mastroianni et al. 2014). Furthermore, there was attenuated Crp 2 and Crp 3 responses against pathogenic *E. coli* strains tested in anoxic conditions in comparison to oxygen-rich conditions (Mastroianni et al. 2014). Some plausible explanations for differential responses to α -defensins based on oxygen presence involve consideration of iron acquisition and sequestration that may trigger induction of lipid A or lipoteichoic acid modification to alter the peptide interactions (Boulette and Payne 2007).

A call for solutions

Although the establishment of ATCC bacterial and fungal strains has facilitated standardization and streamlining of microbiological testing in multiple scenarios, these standardized protocols miss the mark when considering inter-strain variability and endogenous microenvironmental factors that may impact susceptibility data. Testing confined to only a single or few strains, whether ATCC or clinically acquired, can substantially underestimate or overestimate the capacity of a drug to hinder microbial growth. Negligence for strain diversity and the wide array of extraneous factors influencing susceptibility assessments can lead to wrongful interpretation of data that is especially dangerous in the era of MDR. For instance, New York State's Department of Health Clinical Laboratory Standards of Practice for MIC quality control, assessment and recording makes no mention of stressing inter-strain diversity or testing in varying pH, salt levels, and oxygen content for determining MIC (New York State Department of Health. Clinical Laboratory Standards of Practice, Part 2 - Specialty Requirements 2016). Universally employed standardized laboratory protocols, such as CLSI/NCCLS standards for antimicrobial susceptibility testing for antibiotics and antifungals in our current armamentarium, do not apply for testing of experimental drugs making drug efficacy comparisons difficult at pre-clinical stages. This issue mirrors the inconsistency of evaluation criteria for categorization of drug resistance that precludes meaningful comparison of data across countries (Rodloff et al. 2008). A solution to sidestepping this issue is calling on investigators to standardize the

approach in which novel antimicrobials at the level of research and development are tested with emphasis on increasing the numerical strain threshold, with inclusion of well-characterized hypermutating strains, to better account for the vast breadth of microbial strain diversity. By increasing the amount of strains that are required for testing to a threshold deemed adequate and thorough, susceptibility testing can better intercept any inherent weaknesses in a drug that could lead to drug failure at the clinical trial level or that may lead to conferring of resistance after successive use.

Furthermore, Standardizing the inclusion of hypermutator phenotypic strains during pre-clinical drug susceptibility testing has the capacity to showcase iterations of resistance up to 10,000 times that of wild-type strains (O'Neill and Chopra 2001). Hypermutating strains can also be exploited to elicit exceedingly rare drug resistance phenotypes that naturally occur at frequencies as low as 10^{-15} in order to test novel drugs against these hard to come by mutants (O'Neill and Chopra 2001). For antimicrobials that putatively offer more than one bacterial target, testing of hypermutating strains may be especially relevant as expression of MDR alleles are often triggered in certain pathogens challenged by multi-target drugs (LeClerc et al. 1996). Although many of these mutations have shown to be deleterious for survival, repeated fluctuations of stressful and nonstressful environments create genetic bottlenecks that facilitate the sustainability of microbial pathogenicity (Martinez and Baquero 2000). Lastly, hypermutable strains may have acquired this capability via environmentally-regulated transposons, highlighting our previous argument concerning the importance of testing susceptibility in strains in modified environmental conditions (Martinez and Baquero 2000). Incorporation of these hypermutating strains alongside a vast array of clinically acquired strains during susceptibility testing can identify whether an antimicrobial has inherent vulnerability to resistance mechanisms at early stages of development and may expedite the characterization of promising antimicrobial drugs.

As a recommendation for future novel antimicrobial testing, we propose the testing of at least 100 clinically isolated strains of the same pathogen with inclusion of MDR as well as hypermutator strains (if available) at the pre-clinical stage. These recommendations are qualitatively derived via appraisal of aforementioned data concerning strain diversity and its impact on antimicrobial efficacy, in conjunction with quantitative data for use of approximately 100 strains based off of pre-clinical studies of antimicrobial success stories that have been recently approved by the US Food and Drug Administration (FDA) or have anticipated approval for clinical use (Monogue et al. 2017; Andrei et al. 2018). Indeed, the pipeline for introduction of new agents against infectious diseases lags significantly behind other disciplines such as oncology, yet important lessons can be gleaned from the few antibacterial molecules that were recently approved by the FDA for clinical use (Andrei et al. 2018). For instance, pre-clinical studies helping to establish delafloxacin, a non-zwitterionic fluoroquinolone antibiotic with documented broad-spectrum activity, as a viable antimicrobial candidate reinforced the importance of testing a large quantity of clinically acquired strains at earlier stages of development. An earlier study tested delafloxacin's efficacy against at least 100 strains of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, including strains of penicillin-resistant, ceftriaxone-nonsusceptible, and levofloxacin-resistant subsets of *S. pneumoniae*. Delafloxacin was shown to be 128-fold (MIC_{50}) and 64-fold (MIC_{90}) more active than levofloxacin against all *S. pneumoniae* isolates and was noted

to possess enhanced potency when compared to ceftaroline. The MIC₉₀ was 4-fold and 8-fold lower than those for ciprofloxacin and levofloxacin, respectively, when tested against *H. influenza*. Lastly, delafloxacin was the most potent agent tested against *M. catarrhalis* as it exhibited an MIC₉₀ that was 8-fold lower than those for other fluoroquinolones such as ciprofloxacin and levofloxacin (Flamm et al. 2016). The results from this study and similarly robust pre-clinical studies may facilitate the pooling of data that incorporates thousands of strains to expedite validation of promising antimicrobials (Van Bambeke 2015; Pfaller et al. 2017). Ceftazidime-avibactam, an anti-pseudomonal third generation cephalosporin combined with a new broad-spectrum non- β -lactam β -lactamase inhibitor, is another recently launched antimicrobial that underscored the importance of testing a large batch of strains at early pre-clinical stages. For example, 396 *P. aeruginosa* isolates were tested *in vitro* and were demonstrated to possess 67.4% susceptibility to ceftazidime-avibactam with nonsusceptibility to ceftazidime, cefepime, meropenem, and piperacillin-tazobactam (Sader et al. 2015). Follow up studies built upon this data by increasing the threshold for adequate testing into the thousands; 1,743 *P. aeruginosa* isolates treated with ceftazidime/avibactam again demonstrated the highest percentage of susceptible isolates versus comparator β -lactams, with 91.5%–100% susceptibility, second only to the last resort antimicrobial colistin (98.0%–100% susceptible) (Huband et al. 2016). Another exemplar for pre-clinical studies that incorporated at least 100 strains of the same pathogen at early stages of drug development focused on the evaluation of cefiderocol, an innovative siderophore cephalosporin showcasing potency against clinically encountered MDR gram negative organisms (Dobias et al. 2017). In this study, *E. coli*, *K. pneumoniae*, *Enterobacter* sp. and *A. baumannii* specimen studies ranged from nearly 100 to 298 strains per pathogen. The MIC₉₀ of cefiderocol was found to be 32-fold less when compared to ceftolozane-tazobactam, meropenem, ceftazidime, ceftazidime-avibactam, and amikacin (Dobias et al. 2017). Of note, cefiderocol is poised to be approved by the FDA in the setting of multiple studies including a profound diversity of strains illustrated by the inclusion of hundreds to thousands of clinical strains (Dobias et al. 2017; Hackel et al. 2017; Andrei et al. 2018).

Unifying databases and incorporation of microenvironmental parameter algorithms

Currently, a wide array of databases for microbes with complex mechanisms of disease and resistance, including *A. baumannii* and *P. aeruginosa*, exist detailing mutant libraries, sequence analyses, gene annotations and functional pathway analyses that in theory should facilitate drug production that foresees impediments to efficacy. In a similar fashion, bioinformatics approaches have surfaced to help expedite drug discovery across a multitude of disciplines (Ekins, Clark, et al. 2014). Although these mechanisms of “big data” collection help expand our collective understanding of microbial pathogenicity and hypothetically help create a pipeline informing rapid drug design, these databases have had little impact on the acceleration of defining validated targets for resistant infectious agents. Indeed, most new antimicrobials that are currently in the process of advancing to clinical trials are repurposed compounds or combinations of compounds that do not emphasize drug novelty against organisms with startling levels of resistance such as the ESKAPE pathogens (Simpkin et al. 2017). Many of these databases exist disparately creating a hindrance to their prudent utility without regard to previous studies; a prime example highlighting this issue is screening for identification of novel drug compounds against *M. tuberculosis* (Ekins, Clark,

et al. 2014). Although there has been an upsurge in screened compounds with as many as 5 million in the past decade, successful hit rates of these screens are abysmal in the low single digits. Part of the problem relates to the scarcity of centralized, large data coordination of *M. tuberculosis in vivo, in vitro* and/or clinical data databases (Ekins, Clark, et al. 2014). As has been previously suggested, circumventing this problem calls for coordination amongst scientists for thoughtful integration and analysis of the overwhelming amount of data at our disposal prior to employing screening algorithms (Ekins, Pottorf, et al. 2014). For the realm of rare and unusual pathogens, MicrobeNet, a newly unveiled online database curated by the US Center for Disease Control and Prevention (CDC) helps characterize undescribed pathogens via genetic sequence using 16S ribosomal RNA sequencing, protein profiling generated by MALDI-TOF MS, phenotyping based on biochemical analysis, morphological characterization as well as antibiotic resistance profiles (MicrobeNet CDC). This platform could be used as a prototype for creation of a pathogen-specific centralized database that houses comprehensive *in vivo, in vitro* and/or clinical data in conjunction with bioinformatics approaches for identification, selection and prioritizing of potential disease targets to better assist drug development. For proper assessment of novel antimicrobial drugs in the context of strain diversity, the creation of comprehensive databases including the expansive gamut of a pathogen's potential resistance mechanisms, morphotypes and virulence factors that can then be stratified based on mutable environmental factors may prove advantageous in drug development.

As clearly evidenced, the milieu a pathogen encounters can severely compromise drug susceptibility or enhance resistance; the relevance of these environmental parameters must not be downplayed in antimicrobial testing at pre-clinical stages. Development and testing of mathematical models similar to those that predict patterns of food spoilage and product shelf-life can play a role in addressing how inter-strain variance impacts antimicrobial effectiveness. A concrete example of mathematical models that could be fitted to pre-clinical antimicrobial testing can be gleaned from studies of *Pseudomonas spp.* on sliced mushrooms stored at temperatures ranging 4 and 28°C that were obtained and fitted to three different primary models, known as the modified Gompertz, logistic and Baranyi models (Tarlak et al. 2018). These models were fitted to accurately correlate quantity of observed organisms versus predicted organisms as a function of temperature change. In a strikingly similar study of *Pseudomonas fluorescens* in fresh meat, the primary Baranyi and Roberts and the modified Gompertz models were fitted to the experimental data to predict growth parameters and the Ratkowsky extended model was utilized for assessing pH and temperature effect on a predetermined growth metric (Goncalves et al. 2017). Baranyi and Roberts models ultimately validated the fitted data showing their potential utility in assessing environmental variables that could affect microbial growth; these same models can easily be applied to microbes in the setting of antimicrobial testing with modulation of an expansive set of microenvironmental parameters (Goncalves et al. 2017).

Conclusion

Drug resistance is an inexorable reality that can preclude further use of potent antimicrobials at our current disposal. As a concerted effort to address the current MDR crisis, innovative anti-infectives must be identified and subsequently tested rigorously with a heavy focus on

characterizing drug susceptibilities across a substantial assortment of strains while manipulating conditions that may directly encroach upon drug efficacy or microbial proliferation (Table 2). By increasing and standardizing the amount of tested strains universally, we may be able to better account for the totality of genotypic and phenotypic permutations that are encountered in clinical scenarios. Testing of a large repertoire of distinctive strains, including those that are pre-defined as MDR via classic drug susceptibility testing methodologies, may hasten our capacity to prioritize drugs and compounds that are less likely to fail against organisms notorious for developing resistance. Furthermore, in this calculation, addition of hypermutable strains with characterized “resistomes” offers an option that may also increase the robustness of efficacy data (Martinez and Baquero 2000). To continue optimizing our understanding of newly tested compounds against infectious agents, repurposing mathematical models that have been traditionally used for defining spoilage parameters in food products may provide a more comprehensive understanding of antimicrobial efficacy in a particular microenvironmental niche (Table 2). Understanding differing antimicrobial action across certain unique strains and milieus can only function to better inform drug development in the realm of infectious disease, an arena that has been neglected in recent decades (Aminov 2016). The inclusion of a diversity of strains and modulation of environmental conditions for testing of new therapeutics against infection is far from an original concept but it must be standardized for reliable interpretation of data as we attempt to increase our arsenal against drug-resistant pathogens.

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Table 1.

Factors that influence pre-clinical drug efficacy testing.

Factor	Description
Biofilms	<ul style="list-style-type: none"> • Mechanical barriers for antibiotic penetration • Heterogeneous responses to antimicrobials with documented efficacy against planktonic phenotype • Presence of antibiotic inactivating enzymes and efflux pumps • Capacity to form synergistic biofilms during polymicrobial infection that confer enhanced resistance
Antibiotic Resistance Mechanisms	<ul style="list-style-type: none"> • Hypervirulence conferring resistance across wide-range of antibiotics • Additive mechanisms of resistance (e.g. pre-existing resistance makes it easier to acquire resistance to another antibiotic) • Multi-drug resistance developed by microorganisms without documented previous antibiotic exposure • Isolation of several MDR strains of the same organism from individual patients, with each strain harboring unique resistance mechanisms • Heteroresistance or the presence of several strains from the same organism demonstrating inconsistent susceptibility to an antimicrobial drug • Modulation of virulence upon exposure to an antibiotic that worsens severity of disease
Endogenous microenvironmental parameters	<ul style="list-style-type: none"> • Temperature • pH • Salt conditions • Oxygen content

Table 2.

Recommendations to account for inter-strain diversity when testing potential antimicrobials at pre-clinical stages.

1. Centralizing/Linking of large databases documenting microbial mechanisms of disease and putative drug targets, as well as <i>in vitro</i> , <i>in vivo</i> , and clinical data
2. Standardizing the inclusion of a vast number of clinical strains, along with hypermutator strains, during testing to better approach and capture diversity of strains in clinical scenarios
3. Testing of microbial strains while modulating microenviromental parameters that could affect pathogenicity and/or accuracy of drug testing (i.e. pH, oxygen content, salt conditions, etc)
4. Development of mathematical models that can predict pathogen growth and persistence while modulating microenvironmental parameters in the context of testing an antimicrobial of interest
5.

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