Using Redox Sensitive Dye for

Expedited Antibiotic Minimum Inhibitory Concentration Analysis of

Tier 1 Bacterial BSAT

by

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ABSTRACT

Here, we present an optimized colorimetric broth microdilution assay for *Bacillus anthracis* and *Yersinia pestis* that includes the redox sensitive dye resazurin added to the media. We found the assay results were easier to interpret and gave results significantly faster than the gold standard broth microdilution assay outlined in the CLSI guidelines. We obtained results in roughly half the time; 5h instead of 24h for *B. anthracis* and 28h instead of 48h for *Y. pestis*. Among 32 strains of *B. anthracis* tested with three different antibiotics and 25 strains of *Y. pestis* tested with 4 antibiotics, the assay provided categorical agreement of 100% and 99.7%, respectively. Essential agreement was the same for both bacteria, 99.3%. We also eliminated MIC determination subjectivity by measuring the color shift using a color detection app via smartphone and creating an equation to interpret the results.

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LIST OF ABBREVIATIONS

BMD	broth microdilution
BSAT	biological select agents and toxins
CDC	US Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
DMSO	dimethyl sulfide
Etest	Epsilometer test
MHB2	Mueller Hinton broth II
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MIC	minimum inhibitory concentration
UCC	Unified Culture Collection
USAMRIID	United States Army Medical Research Institute of Infectious Disease
WST	water-soluble tetrazolium salts
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

INTRODUCTION

Antibiotic Susceptibility Testing

Antibiotic resistance is a global concern as antibiotic resistant bacterial strains continue to propagate around the world. Minimum inhibitory concentration (MIC) information allows doctors to create a more effective treatment plan, aiding in antibiotic stewardship and reducing the reliance on empiric therapy. Previous work has shown that access to MIC data improves patient outcomes (Livermore et al. 2012) while delays in effective antimicrobial use increases patient mortality rates (Kang et al. 2003). Therefore, faster results when investigating antibiotic resistance of a bacterial infection is desired in the medical community.

Current methods used in point of care settings to determine MIC include disc diffusion, Epsilometer test (Etest), and microbroth dilution methods. All are based on the simple principle of growing bacteria in the presence of antimicrobials and seeing which ones inhibit bacterial growth. In the disc diffusion method bacteria is streaked evenly across an agar plate and small disks of antibiotic are pressed into the agar surface equidistant from one another. After sufficient bacterial growth (15-48 hours depending on the species), the inhibition area around the discs are measured and susceptibility determined based on inhibition zone size (Bayot and Bragg 2020). Etests are similar to the disc diffusion method, but instead of a disc of antibiotic a strip with an antibiotic concentration gradient is pressed into the agar surface (van Belkum and Dunne 2013). The concentration gradient causes the inhibition ring to take on a tear drop shape, and the bottom point of the inhibition ring is considered the MIC (see image in Figure 1). In contrast, the broth microdilution (BMD) method requires bacteria be grown in a 96 well

plate containing a 2-fold dilution series of antibiotic. Visual observation of bacterial pellet formation or well turbidity is made 24-48 hours later (depending on the bacteria species) to judge growth and identify the concentration at which there was successful inhibition (CLSI 2018). The BMD is currently considered the gold standard of antibiotic susceptibility testing and is currently used as the reference standard for other susceptibility tests (van Belkum and Dunne 2013).

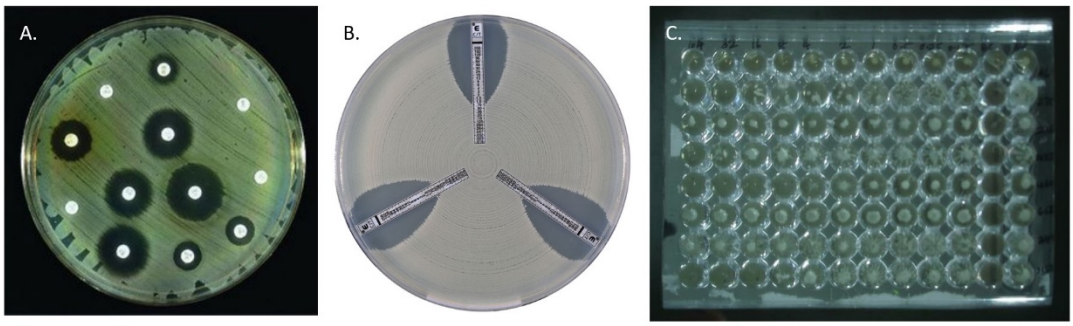

Figure 1: Antibiotic susceptibility testing methods. A. An example of a disc susceptibility test (sciencedirect.com). B. An example of an Etest (biomerieux-asean.com). C. An example of a broth microdilution plate (researchgate.com).

All the previously mentioned methods require similar sample preparation and time to obtain results. Bacterial cultures can be isolated from many types of clinical samples including blood, urine, or swabbing of a lesion. The sample is streaked onto an agar plate to propagate the bacteria, and single, isolated colonies are selected to create a purified sample. These methods all requires relatively high inoculum concentrations, so the purified sample may need to be further propagated in broth or on an agar plate prior to standardization to a concentration of McFarland 0.5 (1×10^8 CFU/mL) for use in the assay (van Belkum and Dunne 2013; Bayot and Bragg 2020). All these assays are also relatively low cost and do not require specialized machinery.

One possible solution to more rapidly assess antimicrobial resistance is to incorporate a redox sensitive dye into the BMD method. A redox sensitive dye exhibits a color change when reduced by NAD(P)H produced during glycolysis (Stockert et al. 2018), this color change is often dramatic and can be observed with the naked eye. We propose introducing a redox sensitive / colorimetric dye component to the BMD assay will speed up result acquisition by enabling visualization of color change prior to when pellet formation can be observed in the traditional assay.

Redox Sensitive Dyes

A popular family of dyes used in these screening assays are the tetrazolium salts which develop color when reduced by NAD(P)H (Riss et al. 2004). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used in the first tetrazolium salt based assay developed for high throughput screening of cell viability (Mosmann 1983). MTT is a positively charged molecule capable of entering the cell; it forms purple formazan crystals when reduced that are insoluble in water and must be solubilized, typically with dimethyl sulfoxide (DMSO), before absorbance can be measured (Riss et al. 2004). To eliminate the need for this solubilization step, second generation tetrazolium salts were introduced, including MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and water-soluble tetrazolium salts (WST); however, they provide their own challenges as they are largely cell impermeable and require the use of a secondary electron receptor to mediate electron transfer (Figure 1) (Riss et al. 2004; Stockert et al. 2018). Tetrazolium salt based assays are touted for easy to read results (Hawser et al. 1998) and have been shown to provide a

shorter time to results for BMD antibiotic MIC assays with results comparable to the Clinical and Laboratory Standards Institute (CLSI) standard broth microdilution method (Tsukatani et al. 2012); however, there has been evidence of antibiotic activity due to the tetrazolium salts which may make them unsuitable for use with some strains of bacteria (Hatzinger et al. 2003).

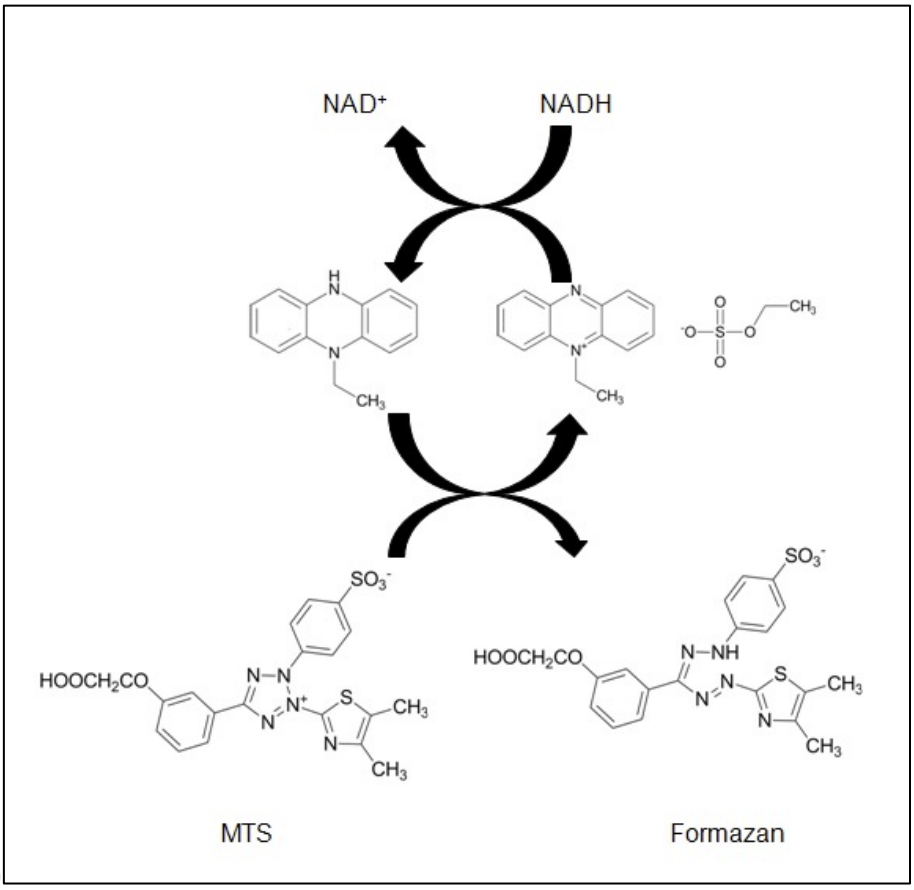

Figure 2: Sulfonated tetrazolium salts require an intermediate electron receptor. Second generation sulfonated tetrazolium salts cannot enter the cell so they require a secondary electron receptor to facilitate reduction (Riss et al. 2004).

Resazurin is another dye proven useful for viability assays. Resazurin is reduced to resorufin intracellularly, resulting in a color shift from blue to pink (Figure 2), and can be can be further reduced to dihydroresorufin, which is colorless (Riss et al. 2004). Resazurin reduction assays are easier to interpret than tetrazolium reduction assays due to

a more dramatic color shift and have been successfully implemented in large scale screening assays (Shum et al. 2008; Kim and Jang 2017) and antibiotic MIC assays (Sarker et al. 2007; Foerster et al. 2017). There is evidence resazurin may also have antimicrobial properties against *Neisseria gonorrhoeae* and *Francisella tularensis* which may make it unsuitable for use with some strains of bacteria (Schmitt et al. 2013; Schmitt et al. 2016).

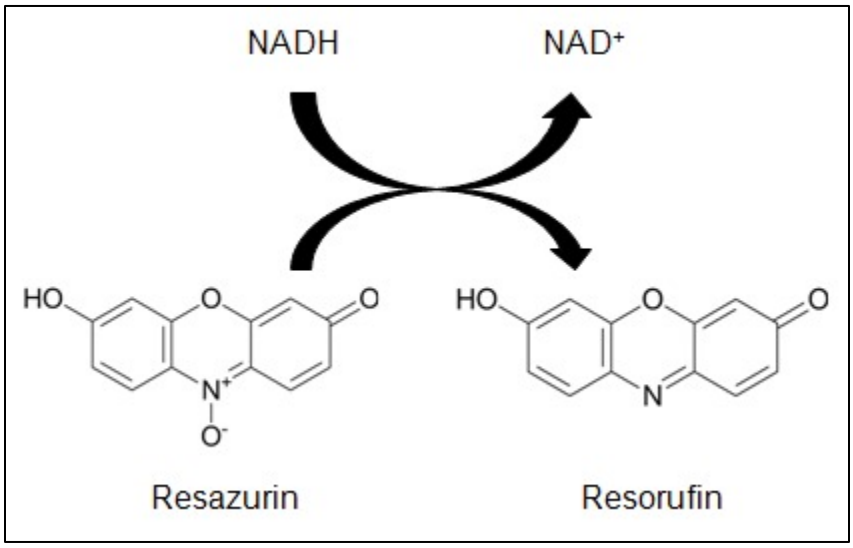

Figure 3: The reduction of resazurin to resorufin. Resazurin is reduced to resorufin in viable cells, resulting in a color shift from blue to pink (Riss et al. 2004)**.**

Biological Select Agents and Toxins

Since introducing redox sensitive dyes to the BMD assay is a relatively new concept, little work has been done testing this technique with bacterial biological select agents and toxins (BSAT). Research with *B. anthracis* and *Y. pestis* is closely regulated by the Federal Select Agent Program, minimizing the number of facilities with access to these agents (Centers for Disease Control and Prevention (CDC), Department of Health and Human Services (HHS) 2017). *Bacillus anthracis* and *Yersinia pestis* are of particular interest due to their potential for weaponization and the health risk presented if naturally occurring antibiotic resistant strains emerge.

Bacillus anthracis is a gram positive, rod shaped, spore forming bacteria which causes an infection referred to as anthrax. *Bacillus anthracis* is found in Central and South America, Africa, Asia, Europe, and the Caribbean, particularly in agricultural areas that do not have a public veterinary vaccination program controlling its transmission among livestock. Humans are typically exposed during contact with infected animals or their tissues; symptoms vary depending on the method of exposure. Cutaneous anthrax results from exposure through a cut in the skin and the patient develops a painless but sometimes itchy ulcer at the site. Inhalational anthrax results in flu like symptoms accompanied by shortness of breath, tightness of the chest, and a cough. Gastrointestinal anthrax from ingestion causes flu like symptoms accompanied by abdominal swelling, vomiting, diarrhea, and a sore throat (Spencer 2003). Anthrax is treated with antibiotics and a vaccine has been developed for pre-exposure use, but is not widely used. Survival depends on treatment and route of exposure. Rapid diagnosis and treatment are important for survival. Even with prompt treatment inhalational anthrax has a 50% mortality (Spencer 2003).

Yersinia pestis is a gram negative, rod shaped bacteria that is the causative agent of plague. It can be found in Africa, Asia, and North and South America. *Yersinia pestis* cycles between fleas and rodent species, occasionally spreading from fleas to humans. Symptoms of plague depend on the route of infection. Bubonic plague results from the bite of an infected flea and manifests as flu like symptoms with extreme swelling of the lymph nodes proximal to the bite. Septicemic plague may result from a fleabite, exposure to an infected animal, or from untreated bubonic plague. Symptoms include abdominal pain, shock, bleeding into the skin and other organs (resulting in the blackened skin often associated with plague), fever, chills, and weakness. Pneumonic plague develops when

bacteria is inhaled leading to flu like symptoms and shortness of breath, chest pain, cough, and may be accompanied by bloody mucus (Pohanka and Skládal 2009). Plague can be treated with antibiotics, and patients who seek medical attention quickly typically make a full recovery; however, if left untreated, bubonic plague is fatal in 50% of cases and pneumonic and septicemic plague are nearly 100% fatal. A vaccine does exist but is no longer commercially available (Rosenzweig et al. 2011).

Both agents are on the US Centers for Disease Control and Prevention's (CDC) Tier 1 Biological Select Agents and Toxins list because they have the potential to cause great harm to human and animal health and have been weaponized in the past (Centers for Disease Control and Prevention (CDC), Department of Health and Human Services (HHS) 2017). While still relatively rare, natural emergence of resistant strains for both bacteria has been documented (Cavallo et al. 2002; Galimand et al. 2006) and many previous works demonstrated ease with which resistant strains can be created in the laboratory (Lindler et al. 2001; Price et al. 2003; Athamna et al. 2004). *Bacillus anthracis* spores are readily found in nature and can be quickly proliferated in the lab; furthermore, spore hardiness lends this agent to weaponization. It has already been used as a weapon several times throughout history, most recently in the 2001 anthrax letters (Pohanka and Skládal 2009). *Yersinia pestis* was weaponized during the cold war but its use as a bioweapon has been documented much earlier using rudimentary methods such as catapulting infected corpses into castles under siege. The high fatality rate from inappropriately treated pneumonic plague makes it extremely dangerous if released in an unsuspecting population.

During a natural outbreak or intentional attack, having access to the fastest MIC assay possible could greatly affect patient care and survival rates. As these agents are

indigenous to areas with limited access to technology and infrastructure, a simple, easy to interpret assay with low resource requirements is ideal. Similarly, military troops stationed outside their home country may be at an increased risk of being targeted by a biological attack, and would greatly benefit from access to quick techniques they can use in the field. Here, we show a redox sensitive dye method can speed up results and simplify interpretation of a broth microdilution assay when determining minimum inhibitory concentrations of antibiotics against *B. anthracis* and *Y. pestis*. We also introduce a novel, highly portable quantification method using a smartphone to aid in assay interpretation.

MATERIALS AND METHODS

Bacterial Isolates and General Culture Methods

Clinical isolates and reference strains were obtained from the Unified Culture Collection (UCC) housed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and resistant isolates from work performed at Northern Arizona University and the Walter Reed Army Institute of Research (Lindler et al. 2001; Price et al. 2003). Isolates were propagated on blood agar (trypticase soy agar with 5% sheep blood) at 35 °C and cultured in Mueller Hinton broth II liquid medium at 35 °C with shaking unless otherwise noted. All *Y. pestis* plates and cultures were incubated at 28 °C. Antibiotics were obtained from Sigma-Aldrich, Thermo Fisher Scientific, or Gold Biotechnology and used at the concentrations given from 100x stock solutions in water. Reference MICs were determined by BMD following CLSI guidelines (CLSI 2018). The susceptibility breakpoints for antibiotics were taken from the 2017 CLSI guidelines (CLSI 2016).

Modified Broth Microdilution

For the standard broth microdilution assay 96-well microdilution trays with round-bottom wells were prepared with 2-fold serially diluted antibiotic in 0.1 mL of MHB2 media. Inoculums were prepared by suspending colonies from fresh agar plates in sterile saline (Remel) to achieve a McFarland concentration 0.5 ± 0.05 McFarland as read by a DensiCHEK Plus (Biomerieux, Marcy-l'Etoile, France). The 0.5 McFarland suspensions were diluted 1:2 with saline, to give suspensions of approximately 5 x 10^7 CFU/mL. Each well in a 96-well plate containing 100 µL of MHB2 and antibiotic mixture was inoculated with 1 µL of bacterial solution and incubated until sufficient growth allowed for visual pellet observation in the positive control well; 24 at 37°C hours for *B. anthracis* and 48

hours at 28°C for *Y. pestis*. MICs were then determined to be the concentration of the well in which no pellet was visible or the pellet appeared <10% the size of the pellet in the control well.

Modified BMD assays were run identical to traditional BMD assays except for the addition of colorimetric dye to the media and a shorter incubation time. Briefly, 96-well microdilution trays with round-bottom wells were prepared with 2-fold serially diluted antibiotic in 0.1 mL of media supplemented with colorimetric dye (see figure 4). Dyes tested included WST-8 (Sigma-Aldrich, St. Louis, Missouri), resazurin sodium salt (Sigma-Aldrich), and XTT sodium salt (Sigma-Aldrich) with phenazine methosulfate (Sigma-Aldrich). These were used from a solution 100-1000x the highest concentration required; stocks of non-pre-dissolved dyes were created by dissolving them in water. Each well in a 96-well plate containing 100 µL of broth antibiotic mixture was inoculated with 1 µL of bacterial solution and incubated until sufficient growth allowed for color development. MICs were then determined as the concentration of the well that did not exhibit the color shift of the control well (visual qualitative interpretation).

Figure 4: An example of a modified broth microdilution plate. Rows contain 9 wells of media, in this case Mueller Hinton broth II containing 0.1 µg/mL of resazurin. The first well to the left of the plate (in the red box) serves as the no antibiotic control showing uninhibited bacteria growth. The following 8 wells contain a 2-fold dilution series of antibiotic with the highest concentration (as listed in the image) to the right side of the plate. The sterne stain is fully susceptible to all the antibiotics tested; whereas the Baci374 strain shown is resistant to ciprofloxacin. Empty rows were left between samples to serve as uninoculated media controls and provide indication if the plate had been unintentionally contaminated.

Quantitative Interpretation of Color by Smartphone

After incubation of microdilution trays using the optimized resazurin modified BMD assay with attenuated strains of *B. anthracis* and *Y. pestis*, the V component of the Y'UV color space was quantified for each well by smartphone camera (Figure 5). A Galaxy S7 (Samsung), G3 (LG), or Pixel 3 (Google) smartphone with the Color Grab application (Loomatix) was used for data capture, with the camera left cell set to the YUV HDTV previewed color conversion setting. With the 96 well plate lid removed, the camera's focusing crosshair within the Color Grab application was lined up with each filled well and the V component of the YUV color code was recorded (Figure 6). This data was converted into a growth or no growth interpretation for MIC determination using the following formula:

X = (1 – control well) / (1 – dilution well)

If X ≥ 0.9, the well is positive for growth.

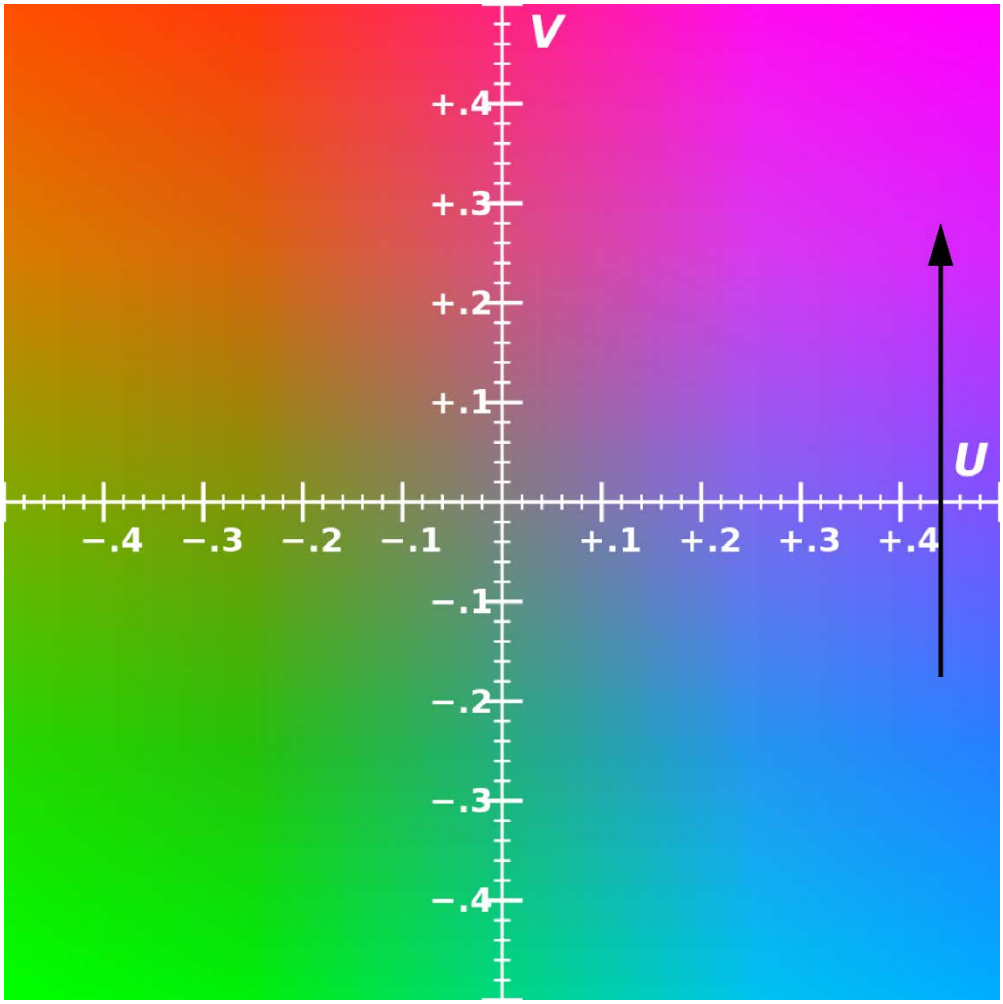

Figure 5: The V component of the YUV color code provides a numeric value which indicates how blue or pink a color is. The v component provides a quantifiable way to measure reduction of resazurin in our assay, an indicator of bacterial growth.

5 hours	A	B- control	C	D	E	F	G	H	I	J	K	L
1		-0.06	-0.06	-0.06	-0.06	-0.06	-0.06	-0.06	-0.06	-0.06	X	
2		0.14	0.07	0.01	-0.02	-0.03	-0.06	-0.06	-0.06	-0.06	Sterne- Cip	
3		0.14	0.01	0.01	-0.03	-0.06	-0.06	-0.06	-0.06	-0.06	Sterne - Pen	
4		0.14	-0.03	-0.05	-0.06	-0.06	-0.06	-0.06	-0.06	-0.06	Sterne- Dox	
5		-0.04	-0.04	-0.04	-0.04	-0.04	-0.04	-0.04	-0.04	-0.04	X	
6		0.17	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.11	374 - Cip	
7		0.17	0.03	0	-0.02	-0.03	-0.03	-0.03	-0.03	-0.03	374 - Pen	
8		0.17	-0.03	-0.05	-0.05	-0.05	-0.05	-0.05	-0.05	-0.05	374 - Dox	

Figure 6: An example of the Color Grab data collected from a modified BMD plate. Color Grab numbers typically ranged from -0.06 for blue, unreduced media up to 0.25 for bright pink, reduced media in no antibiotic control wells where growth was completely uninhibited.

Data Analysis

MICs determined from the resazurin modified BMD assay were compared to those from an unmodified BMD assay as a gold standard. Essential agreement was defined as the percentage of experimental MICs falling within ± 1 two-fold dilution of the standard MIC. In order to determine categorical agreement each MIC was interpreted as either susceptible, intermediate, or resistant based on concentration ranges provided in the CLSI guidelines for each antibiotic and bacteria species combination (CLSI 2016). For strain/antibiotic combinations classified as susceptible or non-susceptible by CLSI, non-

susceptible was treated as resistant for this study. Possible types of errors included minor errors (misclassification of intermediate resistant strains), major errors (false resistant result), and very major errors (false susceptible result).

RESULTS

Dye Selection

We tested 3 different dyes to determine which allowed for uninhibited bacterial growth and provided significant enough color change for gradient readings. WST-8 was tested at 1%, 5%, and 10% concentrations, resazurin sodium salt using 10 µg/mL, 1 µg/mL, and 0.1 µg/mL concentrations, and XTT sodium salt using 10 µg/mL, 1 µg/mL, and 0.1 µg/mL; each XTT concentration was tested with 2 concentrations of phenazine methosulfate, 50nm and 200nm. Each dye concentration was tested with *B. anthracis*, and Y. *pestis*. Upon reduction WST-8 shifts from clear to orange and XTT shifted from yellow to orange, yielding a less obvious color change and minimal ability for gradient readings. We determined resazurin provided the most detectable color change as it shifts from blue to bright pink when reduced and opted to use the lowest concentration of 0.1 µg/mL as it gave equivalent results to the higher concentrations. We were unable to continue experimentation with *F. tularensis* as it requires a growth supplement added to the media containing cysteine. This media reduced all the dyes prior to addition of bacteria, making it an unreliable indicator of bacterial growth for *F. tularensis*.

Assay Incubation Time Optimization

Next, we investigated the effects of adding the dye at different time points throughout the assay. A broth microdilution plate was set up with 8 replicate rows of *B. anthracis* in a ciprofloxacin dilution series. Dye was added to a new row hourly over 7 hours and yielded identical MICs regardless of if the dye was added early or late in the assay. A similar experiment was run with *Y. pestis,* a much slower growing bacteria. Dye was added at the start of the assay and after 16 hours of incubation, again yielding identical MICs under both conditions. Since mixing dye into the media prior to loading it into the

96 well plates saved time and materials with no ill effect on the assay, we chose to utilize that method.

Next, the required incubation time for sufficient color change was determined for each species. We used 3 representative strains each of *B. anthracis* and *Y. pestis* (one susceptible and two ciprofloxacin resistant strains) in a ciprofloxacin 2-fold dilution series. Pictures and observations were taken hourly. Using visual interpretation, we determined the dye significantly reduced in the non-antibiotic control well after 5 hours of incubation with *B. anthracis* and 28 hours of incubation for *Y. pestis.*

To ensure the modified resazurin BMD results matched those from the CLSI standard protocol, we ran the dye and standard assay side by side for a susceptible strain of each bacteria type. We found the redox sensitive dye assay yielded the same results as the CLSI standard assay for both *B. anthracis* and *Y. pestis*. Optimized conditions for a resazurin-based redox sensitive dye are as follows: 0.1 µg/mL of resazurin sodium salt were added to MHB2 prior to loading the plate. Load the plate with media, antibiotics, and bacteria as outlined in the methods for the modified BMD assay. Incubate *B. anthracis* for 5 hours at 37°C or *Y. pestis* at 28°C for 28 hours.

Categorical and Essential Agreement

We collected MIC data in biological triplicate from all accessible strains of *B. anthracis* (32 strains, 18 ciprofloxacin resistant and 14 susceptible) and *Y. pestis* (25 strains 8 ciprofloxacin resistant and 17 susceptible), using our modified assay and comparing to the CLSI gold standard assay. For the 32 strains of *B. anthracis* tested with penicillin 8 µg/mL, doxycycline 16 µg/mL, and ciprofloxacin 4 µg/mL, the assay produced an essential agreement (± a 2-fold dilution of the CLSI results) of 99.3% (286/288 replicates) (Table 1). The 25 strains of *Y. pestis* tested with gentamicin 64 µg/mL, doxycycline 64 µg/mL,

ciprofloxacin 4 µg/mL, and chloramphenicol 128 µg/mL produced a similar essential agreement of 99.3% (298/300 replicates) (Table 2). When MIC results were interpreted as susceptible, intermediate, or resistant, these results translate into a categorical agreement of 100% and 99.7% in *B. anthracis* and *Y. pestis*, respectively, with a single very major error with ciprofloxacin in *Y. pestis*. This error resulted from a false susceptible call for a strain with a reference MIC of 0.5 µg/mL (*Y. pestis* M2 1.106), just above the breakpoint of ≤ 0.25 µg/mL as susceptible.

Table 1: Results of the resazurin based assay with visual interpretation compared to gold standard broth microdilution for *B. anthracis*.

	Ciprofloxacin MIC (µg/mL)		Penicillin MIC (µg/mL)		Doxycycline MIC (µg/mL)	
Strain	Standard	Resazurin	Standard	Resazurin	Standard	Resazurin
ΔANR WT	0.06	0.06 - 0.125	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-1	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-2	0.5	0.5 - 1	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-1	> 4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-2	> 4	≥ 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-3	> 4	≥ 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-1	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-3	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-4	> 4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-5	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-6	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-7	> 4	2 - > 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-8	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-11	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-12	> 4	≥ 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-14	> 4	≥ 4	≤ 0.06	≤ 0.06 - 0.125	≤ 0.125	≤ 0.125
ΔANR S3-15	> 4	≥ 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-16	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-17	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-18	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Sterne	≤ 0.03	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
delta NH-1	≤ 0.03	≤ 0.03 - 0.125	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
New Hampshire	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Vollum	≤ 0.03	≤ 0.03 - 0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Smith 1013	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Texas	0.06	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
K7665	≤ 0.03	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
K6835	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
17T5	≤ 0.03	≤ 0.03 - 0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
K2762	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
K1887	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Ames	≤ 0.03	≤ 0.03	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125

Table 2: Results of the resazurin based assay with visual interpretation compared to gold standard broth microdilution for *Y. pestis.*

	Gentamicin (µg/mL)		Doxycycline MIC (µg/mL)		Ciprofloxacin MIC (µg/mL)		Chloramphenicol MIC (µg/mL)	
Strain	Standard	Resazurin	Standard	Resazurin	Standard	Resazurin	Standard	Resazurin
KIM5 WT	≤ 0.5	≤ 0.5 - 1	≤ 0.5	≤ 0.5 - 1	≤ 0.03	≤ 0.03	2	2
KIM5 M1 1.6	1	≤ 0.5	≤ 0.5	≤ 0.5 - 1	1	1	4	2
KIM5 M1 2.218	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5 - 1	1	1 - 2	2	2
KIM5 M1 1.118	≤ 0.5	≤ 0.5	≤ 0.5	1	1	1	4	2
KIM5 M2 1.106	1	≤ 0.5 - 2	≤ 0.5	≤ 0.5 - 1	0.5	0.125 - 0.5	2	≤ 1 - 2
KIM5 M3 2.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5 - 1	2	2	2	2
KIM5 M3 1.78	≤ 0.5	≤ 0.5 - 1	≤ 0.5	≤ 0.5 - 1	2	2 - 4	2	≤ 1 - 2
KIM5 M4 1.94	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5 - 1	0.5	0.5 - 2	2	≤ 1 - 2
KIM5 M5 1.111	1	≤ 0.5 - 1	≤ 0.5	≤ 0.5 - 1	1	0.5 - 2	2	≤ 1 - 2
pgm-/pPst- parent	1	≤ 0.5 - 1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	≤ 1 - 2
pgm-/pPst- StrepR 10-2	1	≤ 0.5 - 1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- StrepR 10-10	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- GentR 10-8	32	32 - 64	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
CO92	1	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
Pestoides Fmp	≤ 0.5	≤ 0.5 - 1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1 - 2
Kim 10	1	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
PEXU 429	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
Nairobi	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
South Park	1	≤ 0.5 - 2	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
Cambodia	1	≤ 0.5 - 1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2 - 4
Harbin 35	1	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
Pestoides C	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	4	4
T26 mp3	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
25	1	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	4
Pestoides E	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	≤ 1

Resazurin Broth Microdilution Assay in other Bacterial BSAT

We hypothesized the resazurin modified BMD assay may facilitate MIC determination in other biological warfare agents. We did not have access to resistant isolates to perform rigorous evaluation; therefore, we performed an initial evaluation of a few other BSAT bacterial strains. Our findings should be further investigated using more strains with a variety of susceptibilities before determining suitability to the modified BMD assay. We found we could easily distinguish growth versus no growth for most *Brucella spp*. at 24 hours, half the time of the CLSI guidelines for the unmodified assay. We did see a higher rate of error with the *Brucella abortus* BRUC105 strain used (see Table 5). This strain appeared to grow slightly slower than the other Brucella strains as indicated by the less significant color change of the control wells, making it more difficult to determine the MIC at the same timepoint as the other strains. For both *B. mallei* and *B. pseudomallei* the BMD produced 100% essential agreement during initial testing; however, we only observed significant color change after an overnight incubation of 16 hours, while the CLSI guidelines for these species specify a 16-20 hours of incubation. Additives (2% IsoVitaleX) required for *F. tularensis* reduced all the dyes we tested absent bacteria, so a modified BMD assay could not be used with this species, and we were unable to confirm the previously reported antimicrobial activity of resazurin against *F. tularensis* (Schmitt et al. 2013).

Table 3: A comparison of minimum inhibitory concentration for *Brucella spp.* by modified and traditional BMD. With the exception of BUC105, most strains saw essential agreement between the modified and traditional BMD.

	Modified BMD						BMD	
	Round 1		Round 2		Round 3			
Strain	**Gentamicin**	**Doxycycline**	**Gentamicin**	**Doxycycline**	**Gentamicin**	**Doxycycline**	**Gentamicin**	**Doxycycline**
Brucella ovis BRUC020	≤ 0.25	≤ 0.125	≤ 0.25	≤ 0.125	≤ 0.25	≤ 0.125	≤ 0.25	≤ 0.125
Brucella canis BRUC023	1	≤ 0.125	1	≤ 0.125	1	≤ 0.125	0.5	≤ 0.125
Brucella canis BRUC076	1	≤ 0.125	1	≤ 0.125	1	≤ 0.125	0.5	≤ 0.125
Brucella abortus BRUC105	≤ 0.25	≤ 0.125	≤ 0.25	≤ 0.125	≤ 0.25	≤ 0.125	0.5	≤ 0.125
Brucella abortus BRUC106	1	≤ 0.125	1	≤ 0.125	0.5	≤ 0.125	1	≤ 0.125

Table 4: A comparison of minimum inhibitory concentration for *Burkholderia spp.* by modified and traditional BMD. The assay produced a 100% essential agreement amongst the few strains tested; however, little time was saved using the BMD.

	Modified BMD			BMD		
B. mallie	Doxycycline	Ceftazidime	Imipenem	Doxycycline	Ceftazidime	Imipenem
KC238	<.25	1	<.25	<.25	<.5	<.25
KC235	<.25	<.5	<.25	<.25	1	<.25
B. pseudomallei						
unknown	<.25	1	<.25	<.25	1	0.5
unknown	<.25	1	<.25	<.25	1	<.25

Quantification by Smartphone

To remove subjectivity in visually interpreting results we investigated highly portable methods for quantifying the color change of the resazurin dye. The blue to pink color change during reduction falls along the V component of the Y'UV color space and can be measured with the camera on modern smart phones with the use of free color picking applications (e.g. Color Grab by Loomatix). Using the phone's camera, the application outputs a color code while pointed at a well of a microdilution tray without capturing a photo. The V component ranges from approximately -0.1 (blue oxidized form) to 0.25 (pink reduced form).

We found it necessary to develop a formula for interpreting the results that compares the V component color value of each antibiotic dilution well to an antibiotic free control well (see materials and methods). Applying this formula to a data set generated

during the evaluation of the assay with attenuated strains of *B. anthracis* and *Y. pestis*, we acquired identical MIC results compared to visual interpretation in 319 of 345 replicates (92.5%) (Table 5 and 6). Of the replicates with differing MIC results, 19 differed by ± 1 dilution factor and 3 differed by ± 2 dilution factors. There were no classification discrepancies between visual and quantitative interpretation methods. The remaining 4 replicates with differing values resulted from *B. anthracis* ΔANR S3-17, a strain which grows more slowly and to a much lower density than other strains of *B. anthracis* (Figure 7). This lower growth density resulted in low V component color values for the antibiotic free control wells, causing the interpretation formula to behave poorly for this strain. We did not test this phone-based quantification method on fully virulent strains due to limitations bringing equipment in and out of biosafety level 3 containment.

Table 5: A comparison of visual MIC interpretation of the modified BMD assay and interpretation using a color picking application for *B. anthracis* strains.

	Round 1					
	Ciprofloxacin		**Penicillin**		**Doxycycline**	
Strain	**Visual**	**Phone**	**Visual**	**Phone**	**Visual**	**Phone**
ΔANR WT	0.125	0.125	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-1	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-2	1	1	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-1	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-2	> 4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-3	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-1	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-3	> 4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-4	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-5	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-6	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-7	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-8	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-11	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-12	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-14	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-15	> 4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-16	> 4	> 4	≤ 0.06	0.06	≤ 0.125	≤ 0.125
ΔANR S3-17	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-18	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Sterne	0.06	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
	Round 2					
	Ciprofloxacin		**Penicillin**		**Doxycycline**	
Strain	**Visual**	**Phone**	**Visual**	**Phone**	**Visual**	**Phone**
ΔANR WT	0.06	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-1	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-2	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-1	4	2	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-2	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-3	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-1	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-3	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-4	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-5	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-6	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-7	2	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-8	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-11	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125

Table 5 (continued)

ΔANR S3-12	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-14	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-15	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-16	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-17	> 4	> 4	≤ 0.06		≤ 0.125	
ΔANR S3-18	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Sterne	0.06	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
	Round 3					
	Ciprofloxacin		**Penicillin**		**Doxycycline**	
Strain	**Visual**	**Phone**	**Visual**	**Phone**	**Visual**	**Phone**
ΔANR WT	0.125	0.125	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-1	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S1-2	0.5	0.5	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-1	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-2	4	2	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S2-3	> 4	> 4	≤ 0.06	0.06	≤ 0.125	≤ 0.125
ΔANR S3-1	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-3	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-4	4	4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-5	> 4	> 4	≤ 0.06	0.06	≤ 0.125	≤ 0.125
ΔANR S3-6	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-7	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-8	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-11	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-12	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-14	> 4	4	0.125	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-15	4	2	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-16	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
ΔANR S3-17	> 4	> 4	≤ 0.06		≤ 0.125	
ΔANR S3-18	> 4	> 4	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125
Sterne	0.06	0.06	≤ 0.06	≤ 0.06	≤ 0.125	≤ 0.125

*Blue highlighting indicates the MIC could not be determined with the interpretation formula developed.

Table 6: A comparison of visual MIC interpretation of the modified BMD assay and interpretation using a color picking application for *Y. pestis* strains.

	Round 1							
	Gentamicin		Doxycyclin		Ciprofloxacin		Chloramphenicol	
	Visual	Phone	Visual	Phone	Visual	Phone	Visual	Phone
KIM5 WT	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
KIM5 M1 1.6	≤ 0.5	≤ 0.5	1	1	1	1	2	2
KIM5 M1 2.218	≤ 0.5	≤ 0.5	1	1	2	1	2	2
KIM5 M1 1.118	≤ 0.5	≤ 0.5	1	≤ 0.5	1	1	2	2
KIM5 M2 1.106	2	2	1	1	0.5	.25	2	2
KIM5 M3 2.5	≤ 0.5	≤ 0.5	1	1	2	2	2	2
KIM5 M3 1.78	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2	2	≤ 1	≤ 1
KIM5 M4 1.94	≤ 0.5	≤ 0.5	1	1	0.5	0.5	2	2
KIM5 M5 1.111	1	1	1	1	2	0.5	2	≤ 1
pgm-/pPst- parent	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
pgm-/pPst- StrepR 10-2	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- StrepR 10-10	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- GentR 10-8	32	32	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
	Round 2							
	Gentamicin		Doxycyclin		Ciprofloxacin		Chloramphenicol	
	Visual	Phone	Visual	Phone	Visual	Phone	Visual	Phone
KIM5 WT	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
KIM5 M1 1.6	≤ 0.5	≤ 0.5	≤ 0.5	1	1	1	2	2
KIM5 M1 2.218	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	1	1	2	2
KIM5 M1 1.118	≤ 0.5	≤ 0.5	1	1	1	1	2	2
KIM5 M2 1.106	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	.125	.25	≤ 1	≤ 1
KIM5 M3 2.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2	2	2	2
KIM5 M3 1.78	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2	2	≤ 1	≤ 1
KIM5 M4 1.94	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	0.5	0.5	≤ 1	≤ 1
KIM5 M5 1.111	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	0.5	0.5	≤ 1	≤ 1
pgm-/pPst- parent	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- StrepR 10-2	1	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- StrepR 10-10	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- GentR 10-8	64	64	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
	Round 3							
	Gentamicin		Doxycyclin		Ciprofloxacin		Chloramphenicol	
	Visual	Phone	Visual	Phone	Visual	Phone	Visual	Phone
KIM5 WT	1	1	1	1	≤ 0.03	≤ 0.03	2	2
KIM5 M1 1.6	≤ 0.5	≤ 0.5	1	1	1	1	2	2
KIM5 M1 2.218	≤ 0.5	≤ 0.5	1	1	1	1	2	2
KIM5 M1 1.118	≤ 0.5	≤ 0.5	1	1	1	1	2	2
KIM5 M2 1.106	1	1	1	1	0.5	0.5	2	2
KIM5 M3 2.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2	2	2	2
KIM5 M3 1.78	1	1	1	1	4	4	2	2
KIM5 M4 1.94	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	2	0.5	2	2
KIM5 M5 1.111	1	1	≤ 0.5	≤ 0.5	0.5	0.5	≤ 1	≤ 1
pgm-/pPst- parent	1	1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	2	2
pgm-/pPst- StrepR 10-2	1	1	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- StrepR 10-10	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1
pgm-/pPst- GentR 10-8	64	32	≤ 0.5	≤ 0.5	≤ 0.03	≤ 0.03	≤ 1	≤ 1

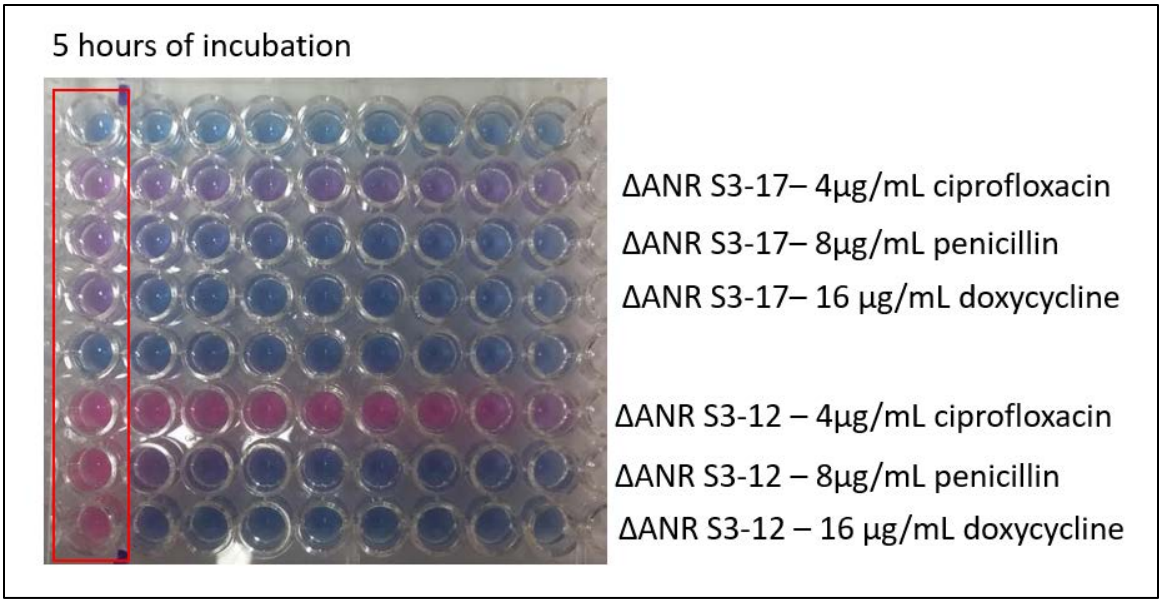

Figure 7: A depiction of the slow growth rate of strain ΔANR S3-17 compared to other strains of *B. anthracis*. This significantly slower growth and dye reduction resulted in an inability to successfully interpret the MIC using Color Grab.

DISCUSSION

Here, we show that the addition of the redox-sensitive dye resazurin to the traditional broth microdilution assay using *B. anthracis* and *Y. pestis* decreases the required incubation time and facilitates interpretation of the results. We generated large datasets of MIC comparisons for *B. anthracis* and *Y. pestis* (Tables 1 and 2) demonstrating that 5 h and 28 h incubations, respectively, are sufficient to differentiate growth from no growth and accurately determine MIC values for these pathogens. With these conditions, we found categorical agreements of 100% for *B. anthracis* and 99.7% for *Y. pestis*, with the single very major error occurring from a *Y. pestis* strain with a reference MIC one doubling dilution above the susceptibility breakpoint. While we did not have sufficient resistant strains to fully evaluate other biological warfare agents, we did find that *Brucella* spp. appear amenable to future development of the assay with an incubation time half that of CLSI guidelines for the gold standard assay. Initial testing of *B. (pseudo)mallei* did not promise shorter time to answer, but the organisms can still benefit from the assay's easier interpretation. We found *F. tularensis* incompatible with any of the redox sensitive dyes we tested due to the requirement for a media additive with a reducing component.

One potential pitfall of BMD assays is the qualitative visual interpretation of the results. Growth cutoff can be difficult to determine if bacteria in wells close to the MIC are marginally inhibited and display much weaker growth, resulting in small pellets (traditional BMD) or less intense pink (resazurin-modified BMD). Additionally, some species and antibiotic combinations display trailing growth with the MIC being considered the first dilution with a cell density of < 10% of the control well, a semi-subjective determination. In the modified assay, this effect manifests as a gradation of pink to purple

to blue colors requiring a cutoff determination for the MIC that is similarly semi-subjective. However, in most cases there is a sharp change in color in the resazurin assay that occurs over either 1 or 2 dilutions, meaning that at most the interpretation would differ by ± 1 two-fold dilution between operators.

One potential solution to ameliorate the issue of qualitative visual interpretation of the resazurin modified BMD assay is incorporating a quantitative method for reading wells that remove operator subjectivity. To achieve this, we assessed the use of a smart phone camera to quantify the V component of the Y'UV color space for each well of a microdilution tray. Through freely available software applications, a smartphone can provide a quantitative color value that can be used in a formula to objectively interpret growth versus no growth. Using this method, we calculated MIC values that aligned well with visual MIC interpretations from both the normal BMD assay and the resazurin modified BMD assay. This strategy removes possible operator bias from results interpretation and eliminates the need to train users on what is "pink enough" to indicate sufficient bacterial growth. Using a smartphone is a much more portable approach to incorporating an objective interpretation system than previous solutions which required larger, specialized instruments such as plate readers measuring fluorescence from resorufin (Foerster et al. 2017). The need for a slightly complex formula for data analysis is a current limitation, but future work with this assay could easily include the development of a smartphone application capable of both recording the color and interpreting the results.

We believe the resazurin modified BMD assay is well suited for use in point-of-care settings for faster susceptibility testing for most bacterial agents. Improved time to answer makes it an appealing method even in modern laboratories or hospitals. Pre-

prepared microdilution trays could easily be fielded to austere locations to help military and civilian populations by enabling operators to evaluate samples of interest with minimal materials or expertise required. Given the need for administering effective therapy as soon as possible after exposure to biological warfare agents, the time savings provided by the faster turn-around-time of the assay and the decreased need for sending samples to a reference laboratory due to reduced equipment requirements make the resazurin modified BMD assay an attractive innovation for antimicrobial susceptibility testing.

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