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Project Title: Screening system development for the rapid detection of cationic antimicrobial resistance mechanisms in Gram-negative bacteria

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Summary (250 words max single spaced):
Background: Cationic antimicrobials (CAs) are positively charged compounds used in hospitals, food industries, and commercial products that work by disrupting bacterial membranes. The inappropriate and increased use of these compounds has promoted to the development of resistance to CA antiseptics/biocides in Gram-negative bacteria. These mechanisms often confer cross-resistance to clinically relevant antibiotics, adding to the alarming rate of antibiotic resistance development. Clinical surveillance for cationic antimicrobial disinfectants are not performed due to the tedious nature of the methods and a lack of standardized drug concentrations.
Objective: To develop a rapid fluorescent-based assay to screen for membrane-based cationic antimicrobial resistance in Gram-negative bacteria
Methods: In the Rapid Fluorescent Dye Membrane Intactness Assay (RFDMIA), we measured the uptake of an impermeant fluorescent dye by Gram-negative bacterial cells in the presence of increasing concentrations of commonly used CAs (colistin, cetrimide, tobramycin). The fluorescent emissions were compared to detect significant differences between CA susceptible and resistant strains at a p-value of <0.05.
Results: The RFDMIA method developed was able to distinguish between the fluorescent emissions of resistant and susceptible E. coli strains for colistin and cetrimide, two well-known CAs (p-value<0.05). Tobramycin was unable to elicit fluorescent dye emissions from tested E. coli strains, suggesting the aminoglycoside was unable to disrupt the bacterial membrane to allow dye entry into the cell.

Student Signature

Primary Supervisor Signature

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1. Introduction

1.1 Background

Bacteria are categorized into two groups based on their membrane structure and Gram-staining potential, those that stain Gram-positive and those staining Gram-negative. Gram-positive bacteria are characterized by a single symmetric membrane externally covered by a thick peptidoglycan layer. Gram-negative bacteria differ by having an additional external layer of protection in the form of an asymmetric outer membrane (OM) (1). This outer membrane not only contributes to antimicrobial resistance (AMR) through its inherent role as a physical barrier, but it can be altered in various ways to decrease susceptibility to antimicrobials (2, 3). Clinically relevant Gram-negative bacteria include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (4).

Antimicrobials have been used as therapeutic and biocidal agents for over 70 years (5). However, bacterial resistance mechanisms have been co-evolving with naturally occurring antimicrobials for billions of years (6). In the past four decades, there has been an increase in clinically isolated antimicrobial resistant Gram-negative bacterial infections (7). The development of new antimicrobial agents is not keeping up with the pace of resistance, threatening a potential health care crisis in the next 50 years if nothing is done (8).

One class of antimicrobial agents where resistance is becoming a growing concern are the cationic antimicrobials (CAs). CAs are positively charged molecules that generally function by disrupting bacterial membranes and cell walls (9–14). The chemical structures of CAs have one or more cationic (permanently charged or pH dependent) charged atoms (typically N, or to a lesser extent P, Ar, As, or Sn) with three to four associated hydrophobic constituents. CAs are used in a wide variety of settings, ranging from wastewater treatment, agriculture, industry, healthcare disinfection, and added to everyday consumer products such as cosmetics and shampoos (2, 10, 13, 14). There are two main types of CAs: 1) therapeutic CAs (polymyxins ;colistin and polymyxin B and aminoglycosides; tobramycin and gentamicin) and 2) biocides/antiseptics (quaternary ammonium compounds (QACs); cetrimide) (Figure 1; 13). The positive charge and lipophilicity of QACs and polymyxins help draw them towards negatively charged bacterial cell walls and membranes, displacing Mg^{2+} ions and lipid interactions (13, 15). Mechanisms of action for CAs involves an initial interaction between positively charged portions of the CA and the negative components of the bacterial membranes such as phospholipids and proteins, which are crucial for maintaining structural integrity, transport of substrates, catabolism and biosynthesis of membrane constituents (11, 13). In the case of polymyxins, Mg^{2+} and Ca^{2+} ions, which are important divalent cations bridging neighboring negative OM lipid constituents, are displaced (15). This interaction is followed by an accumulation of CAs on the membrane, and their subsequent self-assembly into membrane-integrated pores (10, 11, 13, 16). Aminoglycosides, such as gentamicin and tobramycin, are hydrophilic, cationic antibiotics at neutral pH that bind to and inactivate the bacterial 16S ribosomal RNA, impeding protein production (17).

Aminoglycoside antibiotics have multiple methods of entry into Gram-negative cells. For *P. aeruginosa*, currently available studies suggest that aminoglycosides permeate through the OM on their own in a self-promoted uptake mechanism where the drug can permeabilize its own path through each membrane similar as described above for CAs (18). Aminoglycosides have also demonstrated entry into cells through OM porins. (3, 18, 19). At high concentrations of

aminoglycoside cell exposure, these drugs can permeabilize the OM, hence, resistance to these antibiotics may involve multiple membrane alterations (20).

The increased and inappropriate use of CAs in various industries has helped promote the development of a diverse range of AMR mechanisms to these antimicrobial agents (9, 10, 14). For example, QACs reach soils through fertilizer treatment, land waste disposal and sewage sludge (21). Degradation processes are extremely slow in anaerobic conditions, and even in aerobic conditions, QAC degradation is incomplete or slowed down due to adsorption processes which sequester the compounds into layers of clay materials (21). The accumulation of QACs in soils and sediments promotes drug resistance evolution (14). Disturbingly, the CA resistance mechanisms are associated with cross- and co-resistance to other biocides and therapeutic antibiotics (14, 22).

There are three types of AMR: intrinsic, acquired, and adaptive (2, 7, 9, 14). Intrinsic resistance is the ability to resist antimicrobial agents due to an organism's inherent structural and/or functional characteristics (2, 7). This may involve constitutively active efflux pumps and a semipermeable outer membrane, in the case of *P. aeruginosa*[16]. Acquired resistance is the incorporation of foreign DNA in the form of mobile genetic elements (eg. transposons, plasmids) or genetic mutations which may lead to changes in gene expression and/or gene products (2, 7, 9, 14). Adaptive resistance is defined as a temporary decrease in susceptibility to an antimicrobial due to alterations in a gene's expression in response to an environmental trigger such as temperature, nutrient concentrations, or sub-lethal exposures to antimicrobials(7). Adaptive resistance is the only resistance mechanism that is transient and not vertically transmitted to following generations (7).

There are three broad mechanisms of CA resistance: reduced CA uptake due to altered permeability for the antimicrobial through porins, increase in multidrug efflux pump activity to remove CA from the bacterial cell, and lipid modifications that decrease interactions of CAs with the net negative charge of the Gram-negative membrane (2, 3, 7, 9, 10, 14, 17, 24–26) (Figure 2). At the outer membrane of Gram-negative bacteria, CA permeability may be decreased by the reduction of the expression of general diffusion porins, such as the OmpF porin in *E. coli* and *A. baummannii*. Porins are normally used to bring nutrients into bacteria (7). However, changes in size, conductance and/or type of porins may also confer resistance to CAs and other therapeutic antibiotics (2, 3, 7, 13). OM lipid alterations can reduce the net negative charge of the membrane and decrease the CA interaction with and Gram-negative OMs. An important example of this is MCR-1, a lipid modifying enzyme (phosphoethanolamine transferase) that reduces the proportion of negatively charged lipids on the bacterial outer membrane (27). This enzyme confers resistance to colistin, a therapeutic CA of last resort. MCR-1 is transmitted by mobile genetic elements such as plasmids and integrons, increasing the spread of this resistance gene in Enteric bacteria (27). Finally, the third mechanism of CA resistance is the upregulation of intrinsic multidrug resistant efflux pumps such as AcrAB (28) or the acquisition of plasmid/integron transmitted multidrug resistance efflux pumps, such as QacE and QacF, which can promote CA expulsion out of the bacterial cell cytoplasm (26).

E. coli, *P. aeruginosa*, and *A. baummannii* are listed as critical priorities by the World health Organization for research and development due to their high frequency of AMR (4). *E. coli* is highly adept at acquiring multidrug resistant plasmids and mobile genetic elements that confer a variety of AMR genes including *qacE*, *qacF*, and MCR-1 (24–27). AMR among clinical isolates identified in Canadian hospital surveys in the past 5 years (http://www.can-r.com) indicate that

E. coli is the most frequently isolated pathogen in blood and urinary tract infections. *P. aeruginosa* ranks second in respiratory samples (29). *A. baummannii* is a rapidly emerging pathogen that causes nosocomial infections, and is problematic due to its ability to acquire multidrug resistance and to survive under a variety of environmental conditions(30). Higher rates of AMR acquisition by these bacteria poses a significant health threat to communities and healthcare facilities around the world (17).

Currently, clinical surveillance testing for CA biocide resistance is not routinely performed due to the lack of guidelines provided by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (31, 32). DNA-based diagnostic methods, such as Polymerase Chain Reaction (PCR) assays and whole genome sequencing techniques, are used to detect genetic markers of resistance. Predicting AMR based on genetic sequences often fails to identify many CA resistant phenotypes due to the multifaceted nature of CA resistance phenotypes (33). Antimicrobial susceptibility testing procedures, which include agar dilution, disc diffusion, and broth microdilution techniques, are the best and most reliable available options for antibiotic resistance testing (34), however, these methods are tedious and time-consuming. This has led to the need for new standardized screening procedures which can rapidly, accurately and reproducibly discriminate CA susceptible from resistant bacteria.

1.2 Hypothesis

Gram-negative bacteria that demonstrate resistance to CAs caused by membrane-disruptive mechanisms can be rapidly distinguished from other AMR mechanisms by measuring bacterial membrane intactness. To test this, live Gram-negative bacterial cell integrity will be monitored in the presence of increasing amounts of CAs and a membrane-impermeant fluorescent dye will be used to compare CA resistant and CA susceptible Gram-negative bacteria. We expect that bacterial strains with CA resistance mechanisms (either intrinsic, acquired or adaptive) will have a measurable delay in fluorescent emission when compared to those that are CA susceptible. The more resistant a bacterium is to an antimicrobial, the lower we expect fluorescent emission from the impermeant dye to be as it cannot enter the cell and bind to DNA (Figure 3).

1.3 Objectives

The main goal of this project was to develop a rapid screening method to identify CA resistance mechanisms in clinically relevant Gram-negative bacteria that specifically enhance drug resistance by preventing membrane disruption. The method that I participated in developing was named Rapid Fluorescent Dye Membrane Integrity Assay (RFDMIA). RFDMIA will indirectly monitor the membrane integrity of several Gram-negative bacterial strains by measuring the fluorescence emission of a membrane impermeant DNA intercalating dye, as bacteria are exposed to various concentrations of CAs over a 30-minute period. The dye cannot be identified at the present time due to an ongoing patent application (Odd Bress: University of Manitoba Technology Transfer office).

The findings of the RFDMIA were compared to antimicrobial susceptibility testing methods, which determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MIC was defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test, and the MBC as the minimal concentration of drug needed to kill most (99.9%) of the viable organisms after incubation for a fixed length of time (24 hours) under a given set of conditions

(35, 36). For the purpose of our study, the MBC was defined as the lowest concentration of an antimicrobial agent that kills all microorganisms after 30-minutes of exposure to an antimicrobial and no colony formation on non-selective agar medium after 18 hours of incubation.

Four experimental questions were examined for this project:

1. Does the growth phase of Gram-negative bacteria influence RFDMIA results?
2. Can the RFDMIA method detect CA resistant and susceptible strains of the same species?
3. Can the RFDMIA predict aminoglycoside resistance?
4. Can the RFDMIA detect CA resistance between different species?

2. Materials and Methods

I performed the majority of MIC studies and performed RFDMIA for early optimization of the method, as well as RFDMIA experiments involving colistin and cetrimide under the guidance of my supervisor Dr. Denice Bay and MSc Student Branden Gregorchuk. Summer student Michelle Wuzinski conducted the majority of aminoglycoside RFDMIAs and helped perform some of the MIC studies.

2.1 Strains used and strain preparation

A list of strains and plasmid transformants used in this study are indicated in Table 1. *E. coli* K12 BW25113 strains were used from the Keio collection (37). Dr. Nicola Cartwright from the Bay lab prepared the CA-adapted strains *E. coli* BW25113 NCRG30 and NCetRG40 according to the procedure outlined by Bore et al in 2007 (38). Clinical isolates were received from CADHAM Provincial Laboratories (courtesy of Dr. David Alexander, Cadham Provincial Labs). All strains were streak plated overnight (incubated at 37°C for 18 hours) from frozen 12% v/v dimethylsulfoxide (DMSO) stocks on Lysogeny broth (LB) and/or Mueller Hinton Broth (MHB) agar plates. Three biological replicates of each bacterial strain were selected from an agar plate, grown overnight in MHB or LB liquid media at 37°C and subsequently cryopreserved in LB with 12% v/v DMSO at -80°C for eventual testing.

2.2 Preparation of antimicrobials and dyes: Working stock solutions of cationic antimicrobial agents (tobramycin, gentamicin, benzalkonium, cetrimide; 10mg/ml, 10mg/ml, 50mg/ml, and 25mg/ml respectively) were prepared with autoclaved 18 ohms purity MilliQ water (for MIC experiments) or phosphate buffered saline (PBS) (for RFDMIA). The concentration ranges for each antimicrobial agent was tested at 2-fold dilutions from the reported CLSI breakpoints or antimicrobial susceptibility testing experiments we conducted herein.

2.3 Antimicrobial susceptibility testing to determine MIC values: Cultures of Gram-negative strains were inoculated from frozen stocks (-80°C) and grown overnight in LB or MHB at 37°C with shaking. Samples were standardized to an optical density at 600nm (OD_{600nm}) of 1.0 and were diluted to have a final concentration of OD_{600nm} in each well of 10^{-4} and 10^{-5} in each well of the 96-well microtiter plates. The plates were set up with increasing concentrations of each CA to be tested, and bacteria were incubated in 37°C for 18 hours. Each plate had a negative control consisting of either MHB or LB without an inoculated bacterial culture, and a positive control with bacteria and no antimicrobial agent. Growth was measured with a Multiskan spectrum UV-Vis plate reader (ThermoFisher, ON) to measure the OD_{600nm} of 96-well plated cultures. The MIC for each bacterial strain was defined as the lowest tested concentration that prevented measurable growth (OD_{600nm}). These values were used to guide the selection of initial CA

concentrations for RFDMIA testing. All testing was performed with three technical replicates of three biological replicates (n=6) (Table 2).

2.4 RFDMIA and cell viability spot plating assays to determine MBC values: Cultures of bacterial strains were inoculated in triplicate from -80℃ frozen stocks and grown overnight in LB or MHB liquid media with shaking at 37℃. The next day, the bacteria were standardized to an OD_{600nm} of 2.0. Two sets of samples were centrifuged at 14,000 rpm for 2 minutes in an Eppendorf microcentrifuge, after which the supernatant was discarded. Pellets were washed once with 1 mL of sterile PBS and resuspended in 1mL of sterile PBS. One set of PBS resuspended cells were heat-treated by incubation at 121℃ for 30 minutes. This set served as the positive control for maximum fluorescent emission in RFDMIA analyses. The remaining cells were used for CA titrations. All RFDMIA samples were measured in 96-well black walled fluorescent microtiter plates at a final OD_{600nm} = 0.2. Fluorescent microplates had the same final concentration of impermeant fluorescent dye (2µg/mL) in each well and increasing concentrations of CA to be tested as determined by MIC experiments above. Excitation and emission wavelengths were determined based on the properties of the dye and appropriate filters on the instruments as close to these values as possible were selected for fluorescent dye emission detection. Negative controls for RFDMIA were wells lacking bacterial culture (ie. PBS only). The amount of fluorescent dye that penetrated the buffered cells in the presence of each CA concentration was monitored every five minutes over a total of 30 minutes using a fluorescence microplate reader, either a Fluostar Optima (BMG Labtech) or POLARstar Optima (BMG Labtech). Different microplate readers were used to confirm that the emission values and trends were reproducible on different instruments and filter sets. After RFDMIA measurements, samples were spot plated (1µl) onto MHB or LB agar plates and incubated overnight at 37℃ to determine the 30-minute MBC value for a particular strain and CA. Growth of each sample was qualitatively graded on a scale of 0 to 3, with 0 representing no growth (=MBC), 1 minimal/spotty growth, 2 moderate growth, and 3 robust growth. All testing was performed with three technical replicates of three biological replicates.

Fluorescent emission data was plotted using Microsoft Excel, and curves were generated from the relative fluorescence intensity (arbitrary units) values of the fluorescent dye versus increasing CA concentrations or 30-minute time points. A student's T-test (heteroscedastic, two-tailed) was performed to assess statistical significance with p-value cutoffs of <0.05.

2.5 Optimization of RFDMIA method: The optimal concentrations of fluorescent dye and bacteria was determined by comparing the fluorescent emissions of live *E. coli* BW25113 and DNA extracted from *E. coli* BW25113 in PBS. Neither PBS-cell preparation was treated with CA. Increasing fluorescent dye concentrations and increasing cell concentrations were used for optimization. Student's T-tests were performed to determine if emission values between live cells and DNA samples at a given cell density and fluorescent dye concentration were significantly different; p-values of <0.05 were deemed significantly different. These findings were used to guide the selection of concentrations for cells and fluorescent dye in final RFDMIA experiments to detect significant differences between live cells and DNA.

2.6 Comparison of various bacteria growth conditions used in RFDMIA: To verify if the method of bacterial growth (planktonic and colony) influences the ability to differentiate between susceptible and resistant bacteria of the same species, three growth methods were examined by RFDMIA for *E. coli* BW25113 transformed with an empty expression plasmid pMS119EH and MCR-1 gene expressing plasmid pMCR-1. Overnight cultures of each strain were grown to

different phases: 1) stationary phase (16hrs), 2) mid-log phase cultures; grown from overnight cultures, diluted 1/100 and grown to OD_{600nm}=0.5, and 3) resuspended agar plated colonies. RFDMIA was performed below, at and above the colistin MIC determined for *E. coli* pMCR-1 strain (Table 2). A student's T-test was performed to compare the emissions between the two live strains to see if significant differences between each sample could be detected for any of the three growth methods.

3. Results

3.1 Optimal dye and cell concentrations for RFDMIA detection

To optimize RFDMIA, a checkerboard analysis involving increasing concentrations of fluorescent dye, with increasing *E. coli* BW25113 cell concentrations (based on OD_{600nm} value) were performed in fluorescent microplates. DNA extracted from E. coli BW25113 served as the positive control. The findings are shown in Figure 4. It was determined that a dye concentration of 2ug/ml and an OD_{600nm} = 0.2 provided the optimal emission conditions for minimizing dye usage and maximizing signal to noise in emission values. With these conditions, emissions from membrane intact live cells and control DNA are significantly different ($p<0.05$). DNA had emissions that were five-fold higher than that of live cells, as compared to three-fold higher at OD_{600nm}=0.1 and four-fold at a fluorescent dye concentration of 1 µg/mL.

3.2 Growth phase does not significantly alter the ability to detect CA resistance using RFDMIA

To ensure that using stationary phase cultures for RFDMIA analysis were ideal, RFDMIA was performed on the same bacterial cultures grown using three different methods: 1) Direct stationary phase overnight culture measurements, 2) mid-log cultures (where overnight cultures were sub-cultured 1/100 into fresh media to mid-log phase OD_{600nm}=0.5), and 3) colonies grown on agar plate suspended in PBS. The outcome of this analysis is shown in Figure 5, where RFDMIA was able to distinguish between *E. coli* BW25113 colistin resistant (pMCR-1) and susceptible (pMS119EH; empty vector) strains at and below colistin concentrations of <8 µg/mL, the MIC value determined for this strain (Table 2). The stationary phase method 1 detected significant differences ($p<0.05$) in fluorescent emissions between *E. coli* BW25113 and *E. coli* BW25113 pMCR-1 at exposure times of 20-35 minutes. The mid-log method 2 detected significant differences ($p<0.05$) at exposure times of 20-30 minutes. Lastly, the agar plate method 3 yielded significant differences ($p<0.05$) at exposure times of 10-35 minutes. From these findings it was determined that 30 minutes of exposure to colistin maximizes the difference in emissions between live cells and heat-treated dead cells for cultures prepared form three different growth methods to yield similar RFDMIA outcomes; each growth method could identify colistin resistant and susceptible strains based on emission values over time using any of the three growth methods tested. For simplicity, the first stationary phase method of growth was performed in all subsequent RFDMIA analyses due to its simplicity and speed of preparation.

3.3 RFDMIA can distinguish colistin resistant *E. coli* strains from colistin susceptible strains

As shown in section 3.2, we performed a RFDMIA study with *E. coli* BW25113 pMS119EH (colistin-susceptible), *E. coli* BW25113 pMCR-1 (lipid-modified; colistin resistant), and *E. coli* NCRG30 (highly colistin resistant; Table 2) strains. The emissions were plotted over time at 3

different colistin concentrations as outlined in Figure 6. At sub-MIC concentrations of colistin, 0 and 0.25 µg/mL, there were no significant differences in fluorescent dye emission values. At 8 µg/mL, the MIC value of *E. coli* BW25113 pMCR-1, the emissions from the susceptible strain was greater than those from the resistant strains ($p<0.05$) for exposure times of 20-35 minutes (pMCR-1) and 15-35 minutes (NCRG30). At 50µg/mL (above the MIC values for *E. coli* BW25113 and *E. coli* BW25113 pMCR-1), there were no significant differences between the emissions of BW25113 and pMCR-1, while NCRG30 had significantly lower ($p<0.05$) emissions. From this study it was determined that the RFDMIA method could show differences in fluorescent emissions of colistin-susceptible and colistin-resistant *E. coli* strains after 30 minutes of exposure time. As a result, remaining RFDMIA plots (Figure 7-9) are shown at 30-minute timepoints for emission values versus drug concentration for each strain.

3.4 RFDMIA cannot predict aminoglycoside resistance but can predict cetrimide resistance

RFDMIA analysis performed with tobramycin at concentrations at or above MIC values for *E. coli* clinical isolates did not result in fluorescent dye emissions, indicating that our highest tobramycin concentration could not penetrate the cells in 30 minutes of exposure (Figure 7). The tobramycin susceptible control strain *E. coli* BW25113 used in this analysis demonstrated an MBC at tobramycin concentrations at or above 128 µg/mL and did not have a significant increase in emission suggesting that the membrane was not disrupted. Heat-treated positive control samples fluoresced at levels comparable to heat-treated samples in other studies, further suggesting that tobramycin did not disrupt membrane integrity for fluorescent dye cell entry.

RFDMIA analysis of *E. coli* BW25113 and cetrimide-adapted *E. coli* NCetRG40 showed significant differences between emission values at all concentrations of cetrimide after 30 minutes (Figure 8). Although the cetrimide-adapted strain had higher emission values at sub-MIC levels of the susceptible strain, the relationship was flipped at cetrimide concentrations at or above 37.5 µg/mL (MIC of *E. coli* BW25113) and was maintained at concentrations past the critical micelle concentration (CMC) of cetrimide.

3.5 RFDMIA slope of emissions may correspond to MIC values

RFDMIA studies done with *E. coli* BW25113 and *E. coli* clinical isolates 93, 96, 97, and 102, which have the same MIC susceptibility to cetrimide, showed two distinct types of emission curves (Figure 9). *E. coli* BW25113 and clinical isolate 93 exhibited biphasic curves with two different plateaus, while the remaining clinical isolates (96, 97, and 102) exhibited sigmoidal curves, in which a plateau was reached at a cetrimide concentration of 300 µg/mL. Furthermore, when the slopes calculated from these emission graphs were compared to MIC or MBC values, we found that the highest rates of change in emissions occurred at cetrimide concentrations at or just below MIC values, but emissions peaked at MBC values.

3.6 RFDMIA can identify intrinsic cetrimide resistance among Gram-negative bacteria, but cannot be used to compare different bacteria

A RFDMIA study performed with cetrimide against *E. coli* BW25113, *P. aeruginosa* and *A. baummannii* produced curves that may be predictive of MIC/MBC values (Figure 10). Emissions from *E. coli* BW25113 and *A. baumannii* peaked at their MBC values, while *P. aeruginosa* peaked at its MIC value. The emissions also varied considerably between species (both strains

with intrinsic resistance had higher emissions compared to *E. coli* BW25113 (p-value<0.05) at sub-MBC concentrations), suggesting that the three species may have different background levels of fluorescence, and it may be difficult to compare differences in emissions in the presence of same antimicrobial between species. Susceptible reference strains representing the same species should be tested by RFDMIA to verify this.

4. Discussion

4.1 Current capabilities of the RFDMIA

This study confirmed that RFDMIA can differentiate between susceptible and resistant bacterial strain, in particular *E. coli* strains with resistance to colistin and cetrimide (*E. coli* BW25113, *E. coli* pMcr-1, *E. coli* NCRG30, *E. coli* NCetRG40). Susceptibility and resistance could be determined from increased fluorescent emissions caused by dye-DNA interactions in cells exposed to CAs cetrimide and colistin at or above their MIC and MBC values, validating the hypothesis. Strains that were resistant (as shown by higher MIC/MBC values compared to controls; Table 2) had lower RFDMIA fluorescent emission values compared to their susceptible counterparts. *E. coli* BW25113 pMCR-1 had greater resistance to colistin (8 µg/mL) as compared to the control plasmid pMS119EH strain due to the overexpression of a lipid modifying gene Mcr-1, as seen from Figure 6. RFDMIA emissions from *E. coli* pMCR-1strains were distinguishable from the controls (*E. coli* BW25113 +/- pMS119EH) at the same exposure times to colistin at or above the MIC values for *E. coli* BW25113 pMCR-1. Hence, colistin resistance derived from lipid modifying enzyme Mcr-1 can be detected by the RFDMIA method. The mechanism of resistance in the colistin-adapted *E. coli* BW25113 NCRG30 is not known at the present time as whole genome sequencing analysis is pending, but increased emission values were detected for NCRG30 strains at high colistin concentrations (up to 162.5 µg/mL) by RFDMIA indicating that enhanced tolerance to colistin can also be detected rapidly for this strain.

Furthermore, analysis of three different growth methods (1. Stationary phase broth cultures, 2. Mid-log phase broth cultures, and 3. Agar colony resuspension) for colistin susceptible and resistant strains of *E. coli* were able to distinguish fluorescent emissions at increasing concentrations representing MIC and MBC values, as seen in Figure 5. This suggests that all three cell physiologies produced similar colistin susceptibility profiles and highlighted the flexibility of diverse starting bacterial cell culture methods by RFDMIA methodology. Although the first stationary phase method was pursued for the majority of RFDMIA analysis in this project due to its speed and simplicity, the other two growth methods detected resistance/susceptibility as compared to controls.

RFDMIA studies done with *E. coli* BW25113, *E. coli* clinical isolates 93, 96, 97, and 102 resulted in dye emission curves that were not identical, even though all strains had the same MIC value. BW25113 and 93 exhibited biphasic curves, while the remaining isolate emission spectra showed sigmoidal curves. This difference may be explained by variations in the membrane composition, or mechanisms of how drugs bind to and disrupt the membrane which impact the rate of our dye's uptake and fluorescent intensity over time. The sigmoidal curve may correspond to the Langmuir uptake isotherm (L2 subtype) as outlined by Giles et al. in 1960 (39), in which cetrimide molecules adsorb to the bacterial membrane until all the possible interaction sites are filled. The biphasic curve may correspond to the Langmuir L4 subtype, with inflection point at a cetrimide concentration of 150 µg/mL (39).

It is worth noting that in RFDMIA studies involving cetrimide, the fluorescent emission in the heated bacteria (positive maximum emission controls) showed a gradual decrease in emission at higher concentrations of cetrimide > 75 µg/mL. This may be explained by dye and drug quenching interactions, where a decrease in fluorescence intensity of the dye fluorophore due to interactions with cetrimide (40, 41). This interaction could be in the form of direct contact between the dye and the antimicrobial agent at high concentrations, or it could involve the formation of micelles since cetrimide is a detergent that forms micelles at concentrations at or above its CMC (3.88mM = 0.995 mg/mL in water (42)) and phosphate salts are known to reduce the CMC (43). At concentrations above the CMC, membrane vesicle and cetrimide micelles form from the remnants of the disrupted bacterial membrane due to a decrease in surface tension and hydrophobic effects (44). Trapped in micelles, available fluorescent dye is bound and less available to bind DNA and as a result dye emission will begin to reduce as cetrimide micelle formation increases.

RFDMIA did not show a significant difference in fluorescent dye emissions in any of the *E. coli* isolates and control strains we tested at increasing concentrations of tobramycin (Figure 9). *E. coli* BW25113 was used as a tobramycin susceptible control and confirmed the loss of cell viability at 128 µg/mL after 30 minutes of tobramycin exposure, however RFDMIA plots showed no correlation with emissions as we observed colistin (Figure 6) or cetrimide (Figure 7). This analysis adds support to previous studies that suggest that aminoglycosides enter *E. coli* via porins, rather than through the membrane (18). Another possibility is that aminoglycosides, despite causing small disruptions in the *E. coli* membrane, is not destructive enough to create a membrane pore that is large enough to accommodate the passage of our dye (molecular weight ~670 g/mol) across the bacterial cell membrane.

4.2 RFDMIA Strengths and Limitations

The strengths of RFDMIA method lie in its speed and use of fluorescent spectrometry rapidly. Compared to absorbance spectroscopy and flow cytometry, fluorescence intensity measurements in 96 well plates provide a balance between signal sensitivity, speed of collection, and accessibility. Absorbance spectroscopy (Abs), typically used in optical density-based broth culture measurements for determining MIC values, cannot directly or indirectly measure membrane integrity as quickly or as sensitively as fluorescence-based methods, and Abs studies appear to be limited for Gram-positive bacteria with a single membrane architecture. Current Abs methods are limited to Gram-positive species to measure the amount of cellular material leaking from disrupted membranes in the presence of increasing CA (45). If there are multiple compounds/molecules that have the same absorbance wavelength, it is impossible to distinguish leaked material from CA absorption in the background (45). Fluorescent methods have a lower potential for drug-reporter overlap because there is an excitation and emission wavelength that is specific to each fluorophore (40). Flow cytometry has also been used to assess membrane integrity with respect to cationic antimicrobial peptides, also known to disrupt membranes in their mechanism of action (46). Although these techniques are sensitive, flow cytometry is not able to perform high-throughput real-time analysis in under 3 hours, which makes it difficult to visualize how cells change within a rapid time scale. Each time point must be analyzed separately, making the process somewhat tedious and additional cell surface antibodies and dyes are required to determine unbroken and dead cells similar to live/dead assays already commonly in use (47). Flow cytometry can process a small number of

samples concurrently as opposed to the 96 well format of the RFDMIA, that may be expandable to 348 well plates with further optimization.

The weaknesses of the method are also inherent in the use of fluorescent dyes. As discussed above, quenching is a phenomenon that occurs in fluorescent spectrometry and environmental interactions and light scattering caused by cells may impact the intensity and sensitivity of measured signals (40). There is also an issue with standardization of sensitivity across different plate readers which the Bay lab is working to address with a standardization dye plate to account for signal intensity setting differences. The terms “sensitivity” and “gain” are both parameters that help magnify or decrease emission signals in the readers, but the range of their effect on the measured fluorescence intensity is different on each instrument/filter set and requires adjustment. Lastly, the fact that our method targets only membrane-disrupting antimicrobials excludes scores of traditional antibiotics, which act through mechanisms such as inhibition of protein synthesis or cell division. This limits the screening potential to CAs that disrupt the membrane to dye entry.

4.3 Future Directions

The RFDMIA was unable to produce fluorescent emissions in strains against tobramycin. A possibility discussed above is that the membrane disruption caused by the aminoglycoside is not sufficient to allow the entry of fluorescent dye into the cell. To investigate this possible size issue, smaller impermeant dyes could be used. Another option is to use a lipid binding dye, called N-Phenyl-1-naphthylamine (NPN; molecular weight 219.28 (48)), in future RFDMIA studies. NPN fluoresces after binding to hydrophobic acyl chain areas of cell membranes. Fluorescent emission from NPN in previous studies may provide support for the idea that aminoglycosides are indeed creating pores in the membrane that are big enough for NPN but not for our selected fluorescent dye used in this study. In fact, a previous study by Hancock et al. in 1991 has demonstrated increased uptake of NPN following the interaction of aminoglycosides with the outer membrane of *E. coli*, suggesting this may be a useful drug to include for further testing (19).

To prove that CAs actually disrupt cells during RFDMIA experiments, we will need to perform microscopy/electron microscopy studies. Using a confocal microscope or transmission electron microscope, the Bay lab is in the process of comparing the intactness of the bacterial membranes at the same increasing concentrations of antimicrobials used in RFDMIA.

In order to identify specific resistance mechanism that the RFDMIA method can screen for, we have just completed whole genome sequencing of lab adapted CA resistance strains (*E. coli* NCRG30). For strains with multiple resistance mechanisms, RFDMIA can be combined with whole genome sequencing to link phenotypes with genotypes, as well as performing gene deletions to identify CA specific resistance mechanisms.

A CMC study of cetrimide and colistin will be conducted in PBS to determine if the gradual decline in fluorescent emissions of heated *E. coli* cells is due to the formation of micelles.

The RFDMIA methodwe have developed has to date only been used to screen for CA resistance in Gram-negative bacteria, however, Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecium* are also critical priorities as listed by the World Health Organization in 2017 for research and development of new antibiotics (4). Testing for CA resistance in Gram-positive bacteria with the RFDMIA will be undertaken in future studies to increase the applicability of the method.

In addition to improving the RFDMIA standardization for different CAs and Gram-negative bacteria on a single instrument, we must standardize the method across a variety of plate readers. We used microplate readers from 2 different distributors (Biotek and BMG Labtech). The variations in software and hardware between the manufacturers are important considerations in our endeavors to standardize the method across different machines.

5. Conclusions

Statistically significant difference between the fluorescent emission intensities of *E. coli* and other multidrug resistant Gram-negative bacteria to 2 different CAs, cetrimide and colistin, has demonstrated that the RFDMIA method can be used to differentiate between CA resistant and susceptible phenotypes between various Gram-negative bacteria. This is an important step towards improving rapid diagnostic tools for use in other clinical and industrial microbiology and biochemical labs. The methodology has many additional applications to examine the permeability and penetration of membrane disruptive Cas and compare how different AMR mechanisms may differ for currently used and new drugs in development. With further development and optimization, this method could prove to be a quick and efficient means to phenotypically screen for CA resistance. This project will have useful implications for human and animal health, food and wastewater industry surveillance programs, and serve as a tool to help improve more responsible usage of CAs in future food and drug policy development.

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8. Tables and Figures

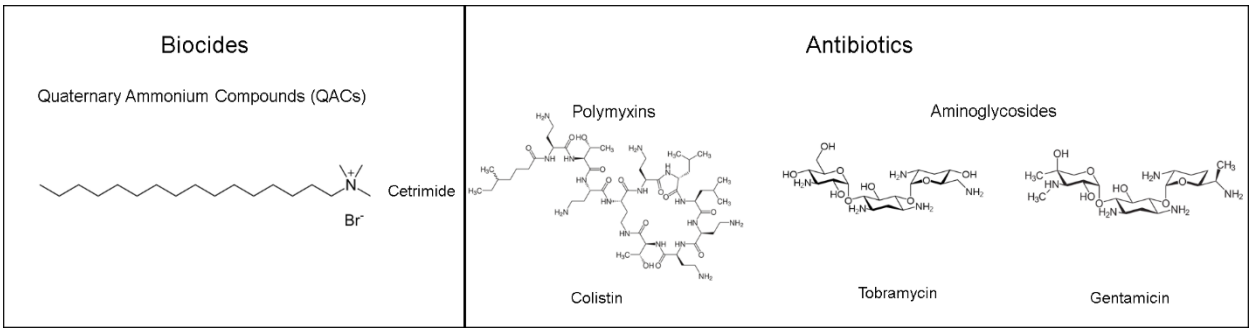

Figure 1. CAs selected for study.

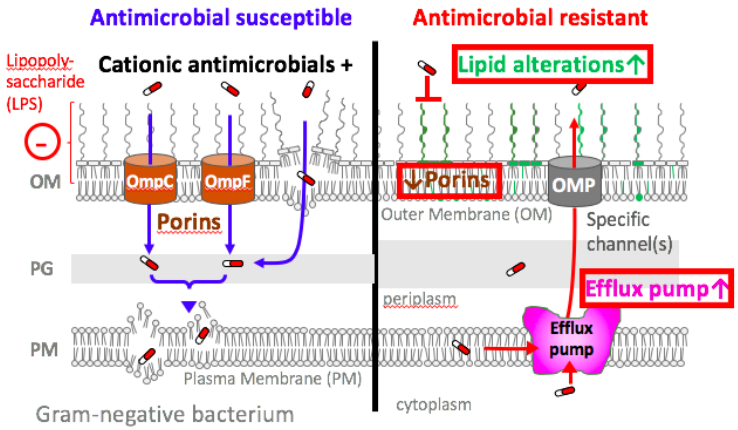

Figure 2. Mechanisms of AMR for CAs (identified in red boxes), which include lipid alterations, downregulation/alteration of porins, and expression of efflux pumps.

Table 1. Gram-negative strains and plasmid transformants used.

Name	Description
***E. coli* BW25113**	Wild-type
***E. coli* BW25113 pMS119EH**	Empty vector
***E. coli* BW25113 pMCR-1**	Phosphoethanolamine transferase gene in pMS119EH
P. aeruginosa PA01	
A. baumannii ATCC17978	
***E. coli* BW25113 NCRG30**	colistin adapted strain after 30 generations of colistin exposure; 400-fold increased resistance
***E. coli* BW25113 NCetRG40**	cetrimide adapted strain after 30 generations of cetrimide exposure; 5-fold increased resistance
E. coli CPL 11198588 (92)	Clinical isolate; Gentamicin resistant
E. coli CPL 17264454 (102)	Clinical isolate; Gentamicin resistant
E. coli CPL 17265024 (103)	Clinical isolate; Gentamicin resistant
***E. coli* CPL 16367516 (92)**	Clinical isolate; Tobramycin resistant
***E. coli* CPL 16403338 (93)**	Clinical isolate; Tobramycin resistant
***E. coli* CPL 17169111 (94)**	Clinical isolate; Tobramycin resistant
***E. coli* CPL 17220070 (96)**	Clinical isolate; Gentamicin + Tobramycin resistant
***E. coli* CPL 17220407 (97)**	Clinical isolate; Gentamicin + Tobramycin resistant

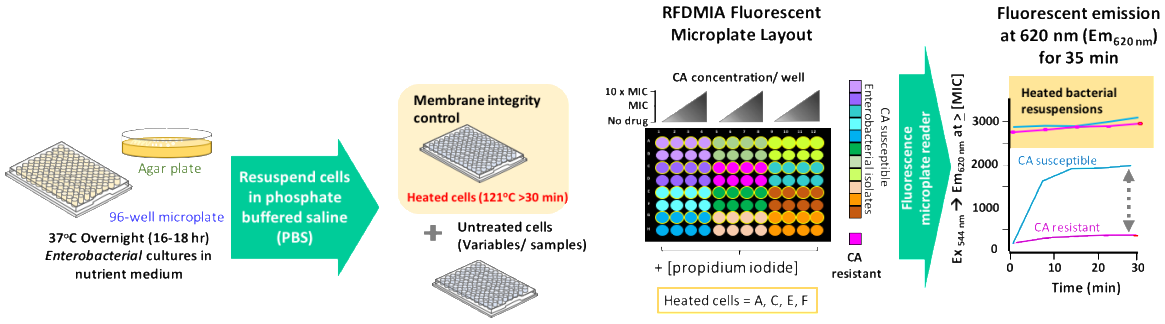

Figure 3. Schematic of RFDMIA testing, with sample microplate layouts and expected results.

Table 2. MIC and MBC values of Gram-negative bacterial strains against tested antimicrobials. MIC values listed with two numbers indicate values obtained at OD_{600nm} of 10^{-4} and 10^{-5}. MIC values listed with an asterisk were obtained by Dr. Nicola Cartwright at OD_{600nm} of 10^{-2}.

Strain	Cet *(μg/ml)* MIC/MBC		Col *(μg/ml)* MIC/MBC		Gnt *(μg/ml)* MIC/MBC		Tob *(μg/ml)* MIC/MBC	
***E. coli* BW25113 No vector**	37.5	75	0.5	8	0.25	-	0.25	128
***E. coli* isolate 91**	37.5	-	-	-	128/64	-	8	
***E. coli* isolate 92**	37.5	-	-	-	128/64	-	32	
***E. coli* isolate 93**	37.5	75	-	-	64	-	32/16	>512
***E. coli* isolate 94**	37.5	-	-	-	128/64	-	16/8	
***E. coli* isolate 96**	37.5	75	-	-	>128	-	128	>512
***E. coli* isolate 97**	37.5	37.5	-	-	32	-	32	>32
***E. coli* isolate 102**	37.5	75	-	-	8	-	32	>32
***E. coli* isolate 103**	18.75	-	-	-	128	-	16/8	-
***E. Coli* BW25113 pMCR-1**	-	-	8	-	-	-	-	-
***P. aeruginosa* PA01**	75	150	4	-	-	-	-	>8
***A. baumannii* ATCC17978**	18.75	75	4	-	-	-	-	>8
***E. coli* NCRG30**	15	-	>200*	>4080	-	-	-	-
***E. coli* NCetRG40**	180*	150	-	256	-	-	-	-

Abbreviations: Cetrimide (Cet), Colistin (Cet), Gentamicin (Gnt), Tobramycin (Tob)

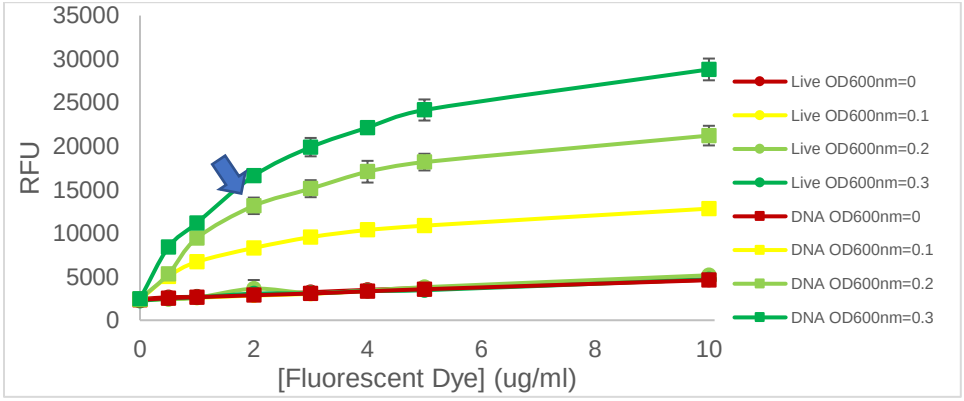

Figure 4. Plot showing the relative fluorescence units (RFU) for *E. coli* BW25113 and genomic DNA extracted from *E. coli* BW25113 at increasing concentrations of impermeant dye and increasing cell concentrations. Each symbol indicates the mean emission value ± standard deviations for three technical replicates of three biological replicates (n=6). Data points indicated by the arrow identifies optimal differences between live bacteria and DNA as well as emission signal to noise, at the lowest amount of fluorescent dye and cells used.

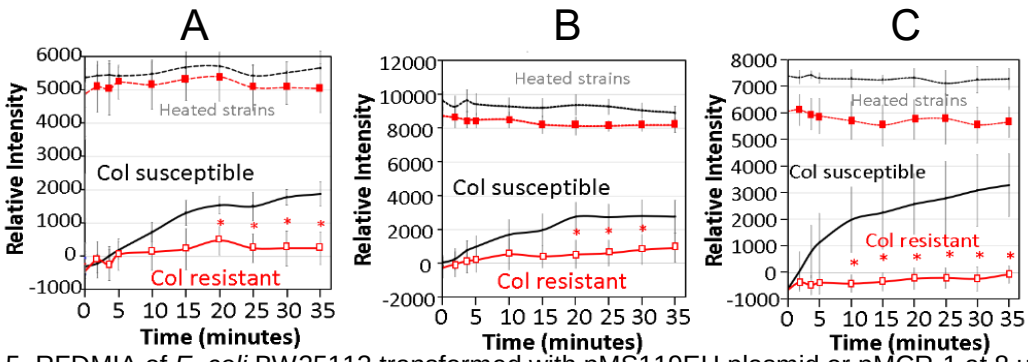

Figure 5. RFDMIA of *E. coli* BW25113 transformed with pMS119EH plasmid or pMCR-1 at 8 µg/ml of colistin (Col) measured using three different growth methods. Stationary phase (A), mid-log phase (B) and resuspended colonies (C) are shown. Black lines indicate col susceptible *E. coli* BW25113 pMS119EH strains and the red lines indicate col resistant E. coli BW25113 pMcr-1 strains. Heated (dashed lines) or live (solid lines) samples are shown. Mean emission values ± standard deviations are shown for three technical replicates of three biological replicates (n=6). Asterisks indicate significantly different emission values at each time point as compared to the col susceptible strain (pMS119EH) with p-values <0.05.

E. coli strain tested	Colistin MIC (µg/ml)
BW25113 pMS119EH	0.5
BW25113 pMCR-1	8
CL adapted BW25113	>200

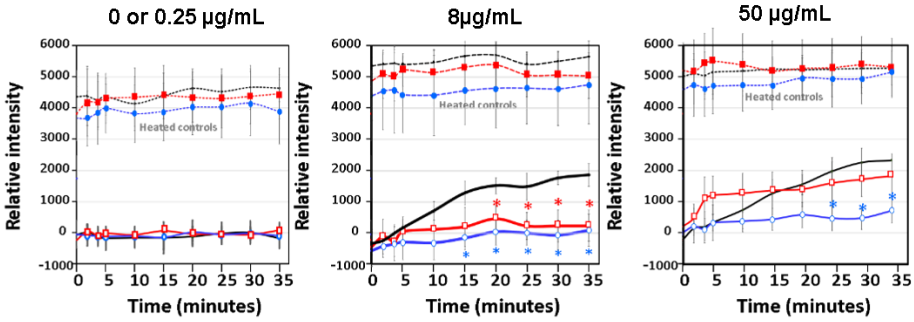

Figure 6. Table of MIC values and three RFDMIA plots of *E. coli* BW25113 pMS119EH, *E. coli* BW25113 pMCR-1, and *E. coli* NCRG30 over 30 minutes at three different concentrations of colistin listed above each plot. Symbols shown in the left-hand table indicates the bacterial samples shown in each of the three RFDMIA plots. The mean dye emission values ± standard deviations for three technical replicates of three biological replicates (n=6) are shown. Asterisks indicate strains where colistin concentrations were significantly different from the control pMS119EH strain based on p-values of <0.05.

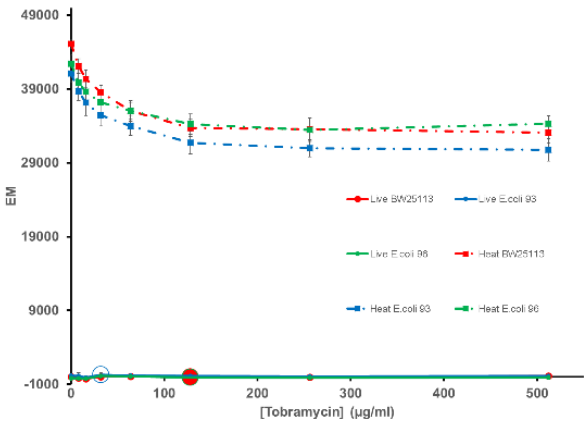

Figure 7. Fluorescent emissions of *E. coli* BW25113, *E. coli* clinical isolate 93, and *E. coli* clinical isolate 96 at 30-minute exposures to increasing concentrations of tobramycin. Each symbol indicates the means ± standard deviations for three technical replicates of three biological replicates. The MIC and MBC values measured for these samples are shown as enlarged unfilled/filled markers on each curve.

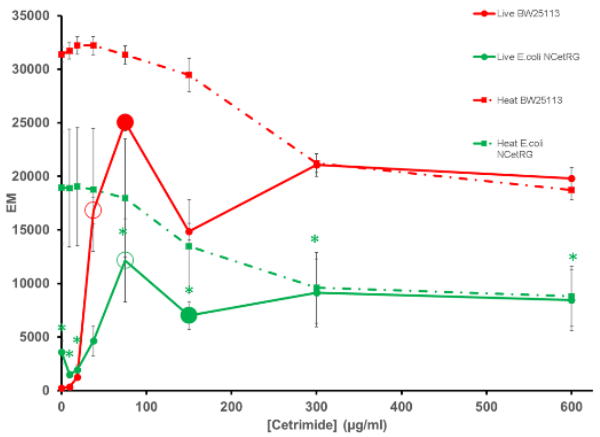

Figure 8. Fluorescent emissions of *E. coli* BW25113 and cetrimide-adapted *E. coli* NCetRG40 after a 30-minute exposure to increasing concentrations of cetrimide. The mean dye emission values ± standard deviations for three technical replicates of three biological replicates (n=6) are shown. Asterisks indicate concentrations where emissions were significantly different from the control BW25113 strain based on p-values of <0.05.

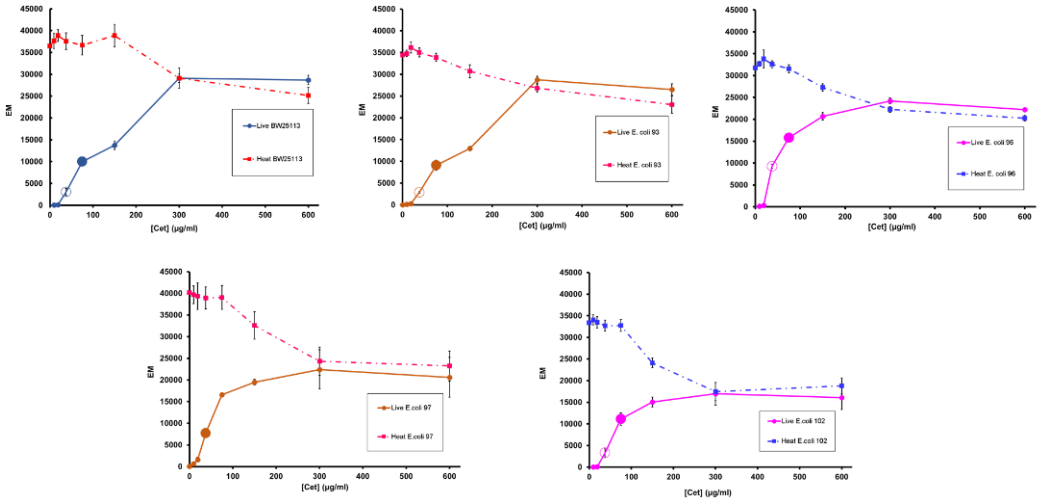

Figure 9. Fluorescent emissions of *E. coli* BW25113 and *E. coli* clinical isolates 93, 96, 97, and 102 at 30-minute exposures to increasing concentrations of cetrimide. Each symbol indicates the means ± standard deviations for three technical replicates of three biological replicates. The MIC and MBC values measured for these samples are shown as enlarged unfilled/filled markers on each curve.

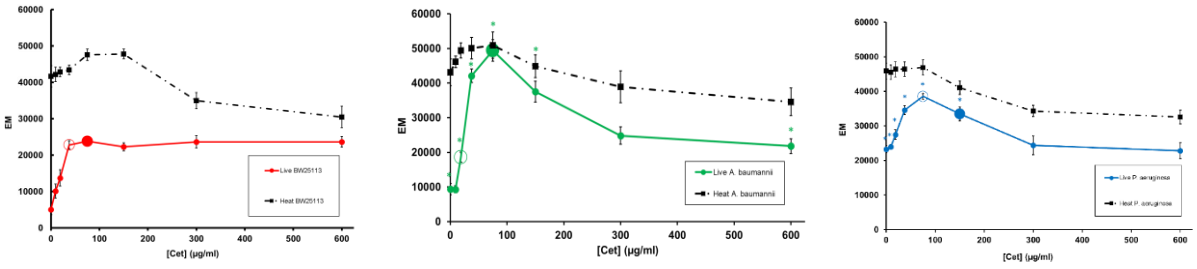

Figure 10. RFDMIA fluorescent dye emissions of various Gram-negative species to determine intrinsic cetrimide resistance. *E. coli* BW25113, *A. baumannii* ATCC17978 and *P. aeruginosa* PA01 were assessed by RFDMIA and emission values after 30-minute exposure to increasing concentrations of cetrimide were measured. Each symbol indicates the mean emission values ± standard deviations for three technical replicates of three biological replicates. The MIC and MBC values measured for these samples are shown as enlarged unfilled/filled markers on each curve. The asterisks indicate significantly different samples as compared to BW25113 control (p-value <0.05).