Direct from Sample Phenotypic Antibiotic Susceptibility Testing

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Master of Science

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by

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The undersigned, appointed by the dean of the Graduate School, I have examined the Thesis entitled

Direct from Sample Phenotypic Antibiotic Susceptibility Testing

Presented by Aiden Lee

A candidate for the degree of Masters of Science in Biological Engineering and hereby certify that, in their opinion, it is worthy of acceptance.

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Table of Contents

List of Figures

List of Tables

Abstract

Background:

To transition from broad-spectrum antibiotic therapy to a more effective "targeted" therapy, i.e. in order to determine whether the infecting strain is resistant or susceptible to individual candidate antibiotics, clinicians and clinical microbiologists need to determine both the pathogen identity (ID) and the Minimum Inhibitory Concentration (MIC) of the candidate antibiotic(s).

It is important to note that the pathogen ID or MIC alone is insufficient to yield clinically significant report for patient treatment. Traditionally, tests to determine both the ID and MIC required use of pure bacterial colonies (obtained by plating the patient samples on agar plates) for testing. However, with recent scientific advancement, there are a number of products developed to determine pathogen ID directly from positive patient samples therefore bypassing the need for colony isolation, and delivering results in approximately 6-8 hours after sample collection. However, doing so for the AST remains a challenge, and so improvements in patient outcome have been limited.

Methods and Findings:

Here, we present a 2-step method to obtain AST-results within few hours of sample collection directly from positive patient blood culture samples (bypassing the need to isolate colonies). The first involves selectively isolating pathogen cells present in samples like blood culture broth using commercially available magnetic-nanoparticles (MNPs) into smaller volumes, thereby bypassing the need for pre-culture. The second involves using microchannel Electrical

Impedance spectroscopy (m-EIS) to monitor multiple aliquots of small volumes (~10µL) of suspension containing bacterial cells, MNPs and candidate-drugs to determine whether cells grow, die, or remain static under the conditions tested. m-EIS yields an estimate for the solution "bulk capacitance" (C_b), a parameter that is proportional to the number of live bacteria in suspension. We are thus able to detect cell death (bactericidal action of the drug) and cell stasis (bacteriostatic action of the drug) in addition to cell-growth. We demonstrate proof-of-principle using *E.coli* and *S.aureus* suspended in blood culture broth. Concentration of bacteria extracted from blood culture broth (using MNPs) was set to be ~100,000 CFU /ml with 10 mg/ml of MNPs in growth culture media. These samples were monitored for the presence of bacteriostatic and bactericidal drugs at concentrations below, at, and above known Minimum Inhibitory Concentration (MIC) values. m-EIS data showed data consistent with growth, death, or stasis as expected and/or recorded using plate counts. Electrical signals of growth and death were visible within 3 hours thus allowing us to perform AST testing within 4 hours.

Conclusion:

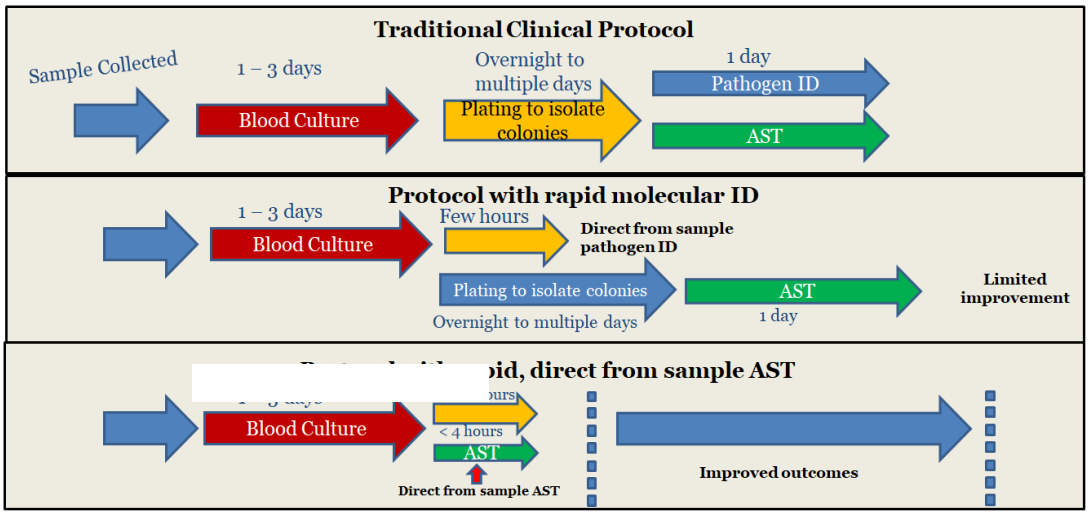
We demonstrated "proof of principle" that (a) CFUs counts can be obtained from positive blood culture and (b) extraction can be performed to isolate the microbial cells to increase its concentration, and (c) that the efficacy of candidate drugs on bacteria thus isolated (in suspensions containing MNPs) could be tested in real-time using m-EIS.

Chapter 1. Introduction

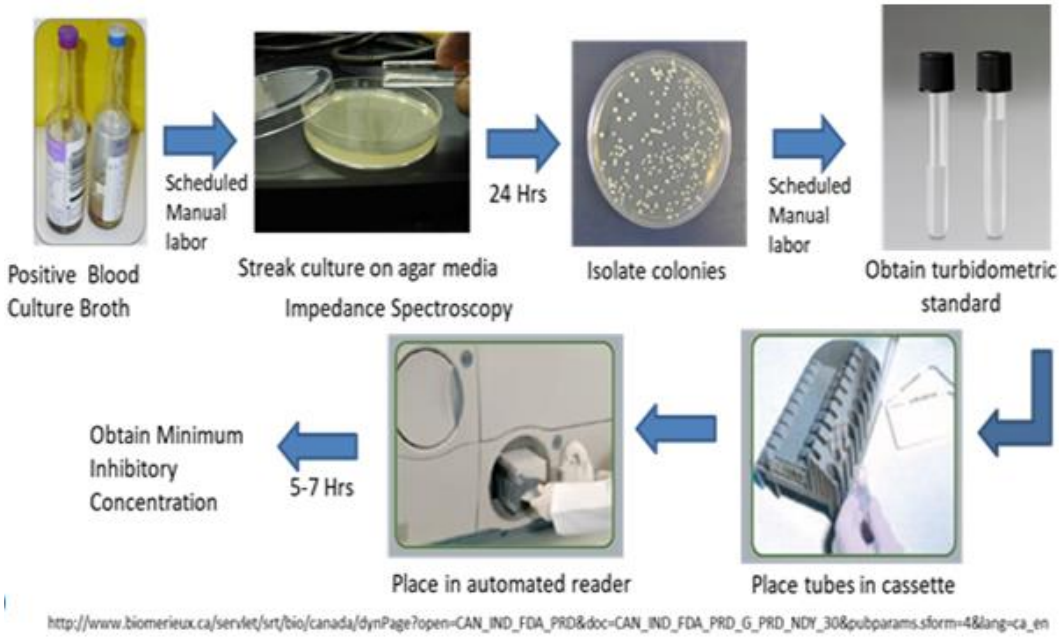
1.1. Motivation

For a large number of infectious diseases including, but not limited to bloodstream infections (septicemia),urinary tract infections (UTIs), respiratory infections (including tuberculosis), infected orthopedic implants, etc., patient outcomes are very closely linked to how quickly clinicians can move from broad-spectrum therapy to more effective “targeted” antibiotic therapy. In some cases, quick transition to directed therapy is a life or death issue. For instance: It has been estimated that for a patient in intensive care suffering from severe sepsis (systemic inflammation brought on by bloodstream infections),the chance of survival drops by an average of ~7% for every hour of delay in switching from broad-spectrum antibiotics (empiric therapy) to an effective antibiotic (directed therapy).[21] In order to deliver effective antibiotics, clinicians need to know both the identity of the pathogen causing the infection and the Minimum Inhibitory Concentration (MIC) of candidate antibiotics against the pathogens to effectively transition from broad-spectrum to targeted antibiotic therapy.

The traditional protocol to obtain both pathogen ID and MIC using instruments like the Vitek™ (Biomerieux) and Phoenix™ (Becton Dickinson) is shown in Figure 1(B). Both ID and AST (MIC determination) involve physically observing bacteria growing (or failing to grow) in specially prepared media: those with different carbon source / nutrients profile for pathogen ID, and when challenged with candidate antibiotics at various concentrations for AST.



(A)



(B)

Figure 1. Timeline and steps involved in obtaining AST results using current protocols

(A) (TOP) Time-line of steps needed to obtain phenotypic Antibiotic Susceptibility Testing (AST results) i.e. values of the Minimum Inhibitory Concentration (MIC) of candidate drugs for a bloodstream infection for three cases. Case 1: using traditional protocol (culture using BACTEC TM / BacT-Alert TM etc., followed by ID and AST using Phoenix TM / VITEK TM, etc.); Case 2: Upon using emerging / recently approved molecular methods for microbial ID (Biofire TM, E-plex TM etc.) after traditional blood culture, while using with traditional methods for AST; Case 3: Upon using proposed "direct from sample" method for AST along with molecular methods for ID. (B) (Bottom): Steps involved in obtaining AST results using current protocols

Because they are optically based, current phenotypic assay systems such as the VITEK (Biomerieux) and Phoenix (BD) require the user to prepare turbidometrically standardized microbial suspensions, and such suspensions are best obtained from colonies, which take from 1 day (over-night) to multiple days, depending on the pathogen, and this greatly delays the start of targeted therapy.

However, in the recent past, there have emerged a number of “molecular” (primarily DNA based) instruments such as the Biofire ™ (Biomerieux), Eplex ™ (Genmark Diagnostics), and Verigene ™ (Luminex). These bloodstream Infection panels are able to determine pathogen ID directly from positive blood culture samples within a few hours, along with information regarding the presence of certain genes that are known to confer drug resistance such as the *mecA* gene for staphylococci or the v*an A/ van B* gene for enterococci or *Klebsiella pneumonia* carbapenemase (KPC).[5,16] While this information is quite helpful for gram positive bacteria (which have a limited range of antibiotic resistant pathways) to guide effective antibiotic de-escalation, in the case of gram negative bacteria, despite being vital in providing genus level identification and the presence of certain important drug resistance genetic markers (E.g. carbapenemases), it does not allow for complete de-escalation in one-step. For example, if it is not a *Pseudomonas*, one can de-escalate from broad-spectrum anti-pseudomonal agents (piperacillin-tazobactam, meropenem or cefepime) to relatively less broad-spectrum beta-lactams (ampicillin - sulbactam, ertapenem or ceftriaxone) and then wait for phenotypic susceptibility results to de-escalate further. The reasons are as follows: In gram negative bacteria the mere presence

of certain resistance genes do not correlate consistently with phenotypic results (E.g. in case of carbapenemases, the level of gene expression is also important) and PCR based methods do not encompass other unknown mechanisms of resistance demonstrated by gram negative pathogens. [14]

Given that the maximal efficacy of antimicrobial therapy is related to maintaining the blood and tissue concentrations above the desired minimum inhibitory concentrations (MIC), it is important for clinicians to maintain steady state concentrations in a clinically improving patient on a given empiric regimen. [12] Such steady-state concentrations are achieved after crossing 4-5 times the half-life of each specific antimicrobial agent (E.g. at least after 3-4 doses for beta lactams and 2-3 doses for each of fluoroquinolones, aminoglycosides and vancomycin).[12] For this reason, switching antibiotics too frequently (first based on PCR results, then based on phenotypic results) is generally not preferred by clinicians (because of the fear of compromising efficacy). Hence, in the case of gram negative infections the clinician may not choose to de-escalate based on PCR results, but rather wait until final phenotypic susceptibility results arrive.

At the same time, one should note that MIC values, on their own, also cannot guide the clinician to targeted therapy with effective antibiotics. Two different pathogens may display the same MIC against a given candidate drug in-vitro. Yet, one of the pathogens may be considered susceptible to the drug, whereas the other may be considered resistant. For instance, according to the guidelines issued by Clinical laboratory Standards Institute (CLSI), a recorded MIC of 4 µg/ml of ampicillin is interpreted differently, depending on the ID of the

organism. Enterobacteriaceae in this case would be considered “susceptible”, *H. influenzae* would be considered “resistant”, and *Streptococcus spp*. would be considered “intermediate”.

However, given that the latest molecular methods now provide pathogen ID results in a few hours, there is now great value to the instruments that can provide phenotypic AST results (MIC values) in a similar “direct from sample” manner in a similar time-frame.

Currently, Pheno system from Accelerate Diagnostics is the only currently FDA approved antibiotic susceptibility system in the market that can go directly from positive blood culture samples to determine antibiotic susceptibility profiles. The positive blood culture sample is processed using automated sample preparation step using gel electro-filtration to separate out the cell debris and other blood cells while retaining the bacteria and yeast cells. This suspension now containing microbial cells in media is tested using their blood culture kit containing reagents and multi-channel cassette. The system uses dark-field microscope to image the immobilized cells in the channels (with each channel containing different antibiotics at a single concentration) over time to obtain time-lapse image of bacterial growth, no growth or lysis. These images are analyzed using their custom software to calculate the MIC values. This system takes 1 hour for bacterial identification and additional 5 hours for AST testing. However, this system is expensive costing ~ $200,000 and can only process one sample at the time and hence has very low sample throughput. Such system will

significantly slow down the clinical workflow when attempting to handle multiple samples.

Thus, the need for a system/method that can provide phenotypic AST results around the same time as molecular methods for microbial ID, inexpensively for a large number of samples at a time, remains unfulfilled. The reported work is an effort to meet this need.

1.2 Proposed Solution

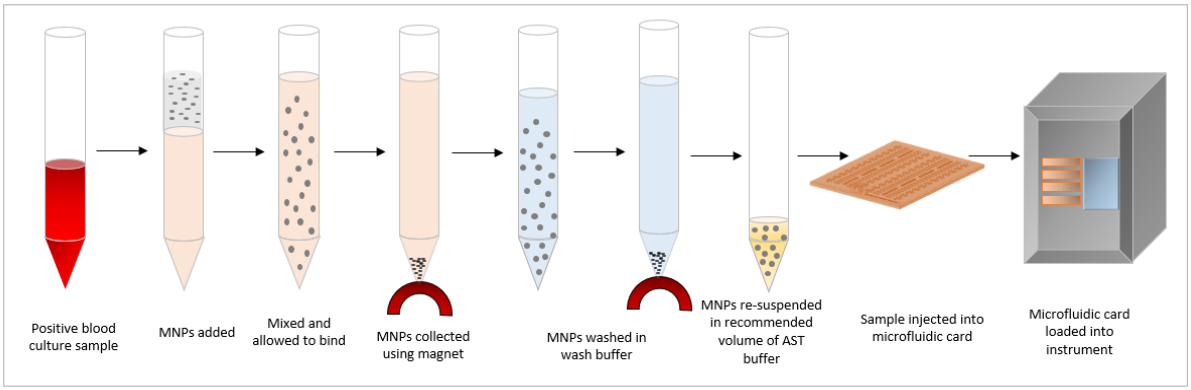


Figure 2. Proposed method for obtaining rapid "Direct from Sample" AST results (MIC values of candidate antibiotics)

The goal of this project is to examine the feasibility of a direct-from-sample phenotypic antibiotic susceptibility testing (AST) approach that bypasses the need for pre-culture and/or obtaining colonies. As shown in Figure 2, this is achieved by first using magnetic nanoparticles (MNPs) to efficiently isolate live pathogen cells from their original matrix (blood culture broth in the current instance) into growth media laced with different amounts of candidate antibiotics, and then monitoring the behavior of the pathogen cells (growth/death/stasis) in real time using Micro-channel Electrical Impedance Spectroscopy (m-EIS) to

determine the Minimum Inhibitory Concentration (MIC) for each of the candidate antibiotics. [19]

Our proposed approach to work includes (a) determining the CFU counts from positive blood culture broth to establish the counts are in a narrow range (b) obtaining good collection/extraction efficiencies from positive blood culture broth using the MNPs and (c) testing the bacterial samples in the presence of MNPs and antibiotics using m-EIS method to determine their antibiotic susceptibility profiles.

Chapter 2. Materials and Methods

2.1 Rationale and overview

MIC values are typically obtained by exposing bacteria at concentrations of ~ 5 x 10^5 CFU/ml to antibiotics at multiple concentrations (usually 10), each a factor of 2 larger than the preceding one. Clinical decisions are made based on MICs recorded at these bacterial loads. It is known that having markedly different loads of bacteria (say 10^8 CFU/ml) can lead to different observed values of MIC. This phenomenon is known as the inoculum effect. Obtaining a turbidometric standard suspension from colonies ensures that the standard concentration of bacteria (5 x 10^5 CFU/ml) is used.

When bypassing the colony formation, we seek to ensure that the suspension that we assay for antibiotic activity has a similar load of bacteria. In this, we are assisted by the fact that at the time blood cultures turn up positive, the number of CFUs present is usually between 10^7 and 10^8 CFU/ml [8]. We first verify that this is true for a limited number of bacterial species. Then we verify our ability to isolate most living cells from that species into a “pellet” using MNPs. This allows us to resuspend the pellet in an appropriate volume so that bacterial loads in the sample subjected to m-EIS are close to the standard.

Once we obtain a suspension containing an acceptable concentration of bacterial cells and MNPs, we seek to show that (a) the presence of MNPs does not affect the ability of the m-EIS method to monitor growth and death of the bacterial cells in suspension, and that (b) the presence of the MNPs does not affect the metabolism of the cells to such an extent that the MIC is altered. So, in

addition to monitoring suspensions containing growth media, appropriate loads of bacterial cells, desired concentration of antibiotic and MNPs, we also monitor suspensions containing all the above except for MNPs.

Our experimental approach is summarized in Figure 3 below,

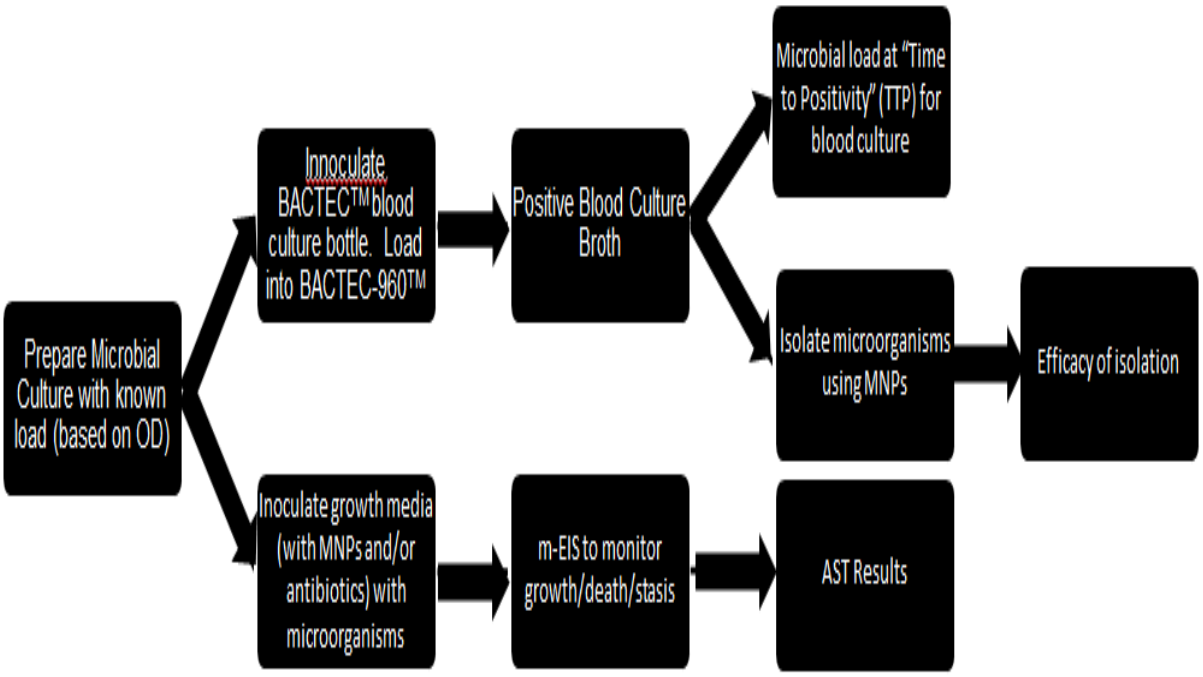


Figure 3. Overview of Experimental Approach

We start by preparing microbial cultures with known loads (CFUs/ml). Using this, "infected" blood is then created to mimic the clinical situation, which is then added to blood culture media (bottle) and incubated in a BACTEC machine to obtain "positive blood cultures". These positive cultures are (a) plated to estimate the microbial load at the time to positivity (TTP), and (b) used to estimate the efficiency with which the MNPs are able to isolate live microbial cells. Also, bacterial cultures with known loads, along with MNPs are used to create simulated versions of cells isolated by MNPs, and these were exposed to candidate antibiotics at various conditions, and monitored for growth or death using m-EIS (along with similar suspensions without MNPs to determine the effect of MNPs, if any) .

2.2 Preparation of Bacterial cultures and seeding of the Blood Culture Broth

Microorganisms used for these experiments were obtained through ATCC and sub-cultured in the lab per instruction material provided by ATCC. Preliminary cell cultures were prepared by growing the cultures overnight. Periodically, their Optical Density (OD) was measured to estimate the concentration of cells. The relationship between OD and cell concentration is a function of the species and the wavelength of measurement. For the species studied, these values (based on previously published literature [2, 4, 11, 15, 20] are summarized in Table 1 below.

Organism (ATCC)	Wavelength in nm	Optical Density	Concentration in CFU/ml
E. coli (25922)	600	0.1	1.60E+08
P.aeruginosa (27853)	600	0.1	1.60E+08
E. faecalis(29212)	600	0.5	1.00E+07
S. aureus (29213)	570	0.15	1.00E+07
K.pneumoniae (700603)	600	0.5	8.00E+08
C. albicans (14053)	520	0.38	1.00E+07

Table 1 Microbial concentrations (CFU/ml) corresponding to measured optical densities (ODs) at selected wavelengths

Once a culture has reached the target concentration, the suspension may is appropriately diluted to obtain a suspension with a concentration of ~500 CFU/ml.

2.3 Finding bacterial loads (CFU/ml) in positive Blood Culture Broth at time of positivity

100 µl of the suspension containing 500 CFU/ml of bacteria are added to ~2ml of human blood (purchased from BioVT) to create “infected” blood. This infected blood is then loaded into BACTEC PedsPlus™ bottles with pre-existing growth media, just like a clinical sample of blood. This sets up cultures of blood with initial loads of ~ 50 CFU/bottle.

The blood culture broth was then sent to the University of Missouri Hospital Microbiology Lab, with instructions to notify us as soon as the BACTEC™ Blood Culture System flagged any bottle as positive. The cultures were brought back to our lab within an hour, serially diluted through 6 orders of magnitude, and 100µl from the three lowest dilutions plated on TSA agar petri-dishes (YPD for yeast). Colonies were counted after 12-48 hours (depending on the species tested).

2.4 Microbial isolation using MNPs

Commercially available Magnetic Nanoparticles (MNPs) [RapiPrep-Micro™ from Microsens Biotechnologies, London UK] were obtained. The beads are provided in a fluidic suspension where the bead concentration is 2.5 mg/ml. These MNPs have been previously used by other researchers to isolate target microbes like *Mycobacterium tuberculosis* from other media like stool and sputum samples. [22, 1], and the purpose of these experiments was to evaluate their ability to extract our model organisms from our matrix of interest (Positive Blood Culture Broth). Positive Blood Culture Broth samples tested were the same ones that were obtained from the previous step.

Largely based on the protocol recommended by the supplier of MNPs, 1ml of the MNP suspension was added to 2 ml of positive blood culture broth. Next, 200 microliters of “capture buffer” (provided by the bead manufacturer) was added to the mixture and it was gently inverted multiple times by the hand for 30 seconds. The mixture was allowed to sit for 10 min with occasional hand mixing (once every 2-3 minutes) in order to expedite the binding of the beads to

effectively capture the cell. A permanent magnet (1" x ½" x ¼" blocks neodymium magnet from Apex Magnets) was then placed along the side near the bottom of the tube. This results in virtually all of the MNPs (both with and without adherent bacterial cells) to accumulate near the magnet to form a pellet, - a process taking about 1 minute.

Using a micropipette, the supernatant was carefully pipetted and discarded. The beads were resuspended in 1 ml of 1X PBS (Phosphate Buffered Saline) and 100 microliter aliquots of resuspension were plated after appropriate dilutions. CFUs at the end of the extraction step were compared to those obtained from plating the initial blood culture broth (before extraction) to get the extraction percentage.

2.5 Sample preparation for m-EIS study

In a real-world situation, we anticipate that the bacteria isolated from human blood using MNPs will be dispersed into growth media, and tested against multiple candidate drugs in parallel. We hence prepared suspensions with bacteria and MNPs in growth media where both the initial load of bacteria (~10^5 CFU/ml) and the concentration of MNPs present (10 mg/ml), were similar to what one would expect to obtain at the end of the isolation process described above in Section 1.3.4. Since it was uncertain / un-verified a-priori if (a) the MNPs would affect the metabolism of cells in a manner that alters their response to the drugs, and (b) the MNPs would adversely affect the electrical measurement, parallel experiments, where MNPs were not added, were also conducted to serve as a baseline when trying to evaluate the effect of the MNPs. For any given

experiments, ~20 ml of suspension (containing bacteria, growth media, and candidate drugs at selected concentrations, with/without MNPs) was prepared, and incubated at 37C. Periodically, 50 µL aliquots were taken from these liquid cultures and subjected to electrical readings.

2.6 Choice of bacterial strains, antibiotics and the concentrations tested

For the in vitro experiments, fast growing organism *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were selected as a representative gram negative and gram positive bacteria, respectively. Both these strains are considered “Quality Control” (QC) strains by the CLSI because their behavior against different antibiotics has been extensively studied. Not only are new products required to demonstrate acceptable results for these bacteria, but clinical microbiology labs are also required to periodically demonstrate the performance of their FDA approved products against these reference strains.

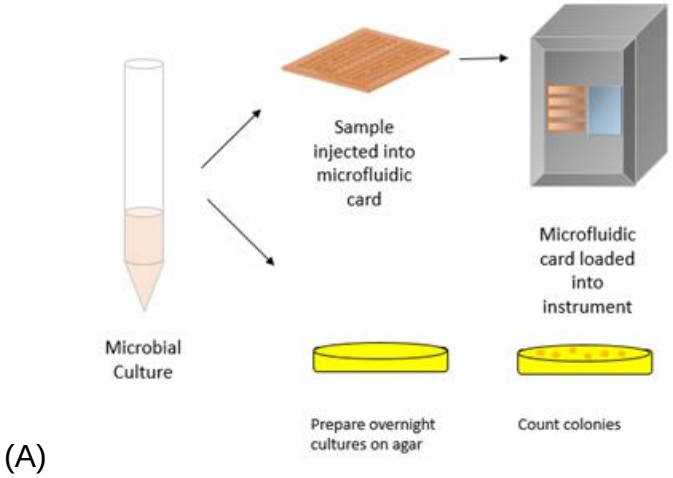
To demonstrate that m-EIS technology can be used to show growth, death, and static response of the cells using MNPs, two antibiotics, ampicillin (bactericidal antibiotic) and chloramphenicol (bacteriostatic antibiotic) were chosen as drug choices for *E coli* and *S aureus* respectively. Antibiotic susceptibility of the *E.coli* and *S.aureus* to the ampicillin and chloramphenicol at varying concentrations and their MIC values were previously shown by others. [10] For *E coli*, ampicillin was chosen as the test antibiotic, which is bactericidal in nature with a known MIC range of 2 - 8 µg/ml [10] and for *S aureus*, chloramphenicol was selected as the test antibiotic which is bacteriostatic in nature with a known MIC range of 2 - 8 µg/ml [10].

As shown in Table 3 and 4, the antibiotics were chosen to represent the different effects that the antibiotics can have with respect to microbial survival: viz. bactericidal (where the drug kills the microorganism) and bacteriostatic (where the net number of live microorganisms does not change). For each cidal/static antibiotic-bacteria combination, whole recommended range of ampicillin and chloramphenicol were tested.

Both *E.coli* and *S.aureus* were cultured in tryptic soy broth (TSB) media (Sigma Aldrich, St. Louis, Missouri*)* overnight with an incubation temperature at 37ºC and continuous agitation. Adjusting the concentration of the bacteria solution was done using UV-VIS spectrophotometer (Azzota S M 1000) to obtain the optical density (OD) of the samples at 570 nm and 600 nm. Literature states that known OD_{570} values of 0.15 corresponds to $1x10^7$ CFU per milliliter for *S.aureus* and OD_{600} values of 0.1 corresponds to 1.6×10^8 CFU per milliliter for *E.coli*.

In a real-world situation, we anticipate that gram positive and gram negative bacteria (like *S.aureus* and *E.coli* respectively) isolated from the patient sample to be tested against various antibiotics to obtain MIC of the infecting strain. Therefore, we prepared suspensions with *S.aureus* and *E.coli* and MNPs in growth media where both the initial load of bacteria ($\sim 10^5$ CFU/ml) and the concentration of MNPs present (10 mg/ml), were similar to what one would expect to obtain at the end of the testing process. Since it is uncertain that (a) the MNPs would affect the metabolism of cells which can alter its response to the drugs, and (b) the MNPs would interfere the electrical measurements, parallel

experiments, where MNPs were not added, were conducted to serve as a control to evaluate the effect of the MNPs.



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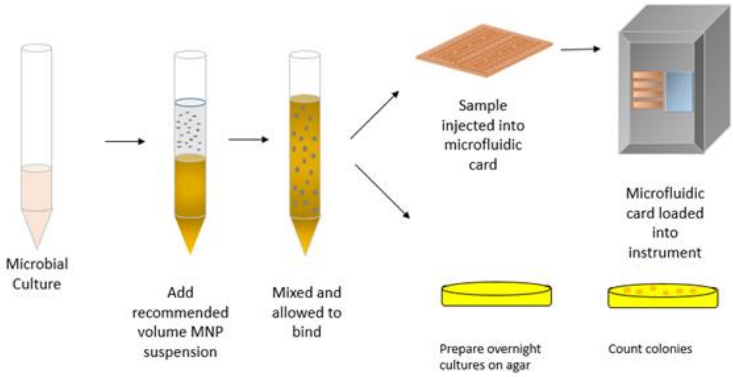


(B)

Figure 4: Experimental Approach using MNP and m-EIS

A) (TOP) Microbial culture (without MNPs) was prepared and the sample was loaded into the cassettes to measure electrical reading. This solution does not contain MNPs and is used as a control.(B) (Bottom): MNPs were added to microbial culture and electrical reading were taken. The m-EIS data obtained from this solution was compared with the control to ensure that electrical reading wasn't affected.

2.7 Electrical Readings

2.7.1 m-EIS

Our lab has previously developed and patented [19] a novel method to monitor the proliferation or death of microorganisms in suspension, which we refer to as microchannel Electrical Impedance Spectroscopy (m-EIS). It relies on the fact that, in the presence of high frequency alternating current (AC) electric field, the membranes of cells become polarized and store charge; thereby acting like electrical capacitors [3]. These capacitances at the individual cells contribute to the overall "bulk capacitance" of the suspension, or net charge stored in the interior. The amount of charge stored by a bacterium is about 100X of that equal volume of aqueous solution [7]. Therefore, even at low concentrations (volume fractions), bacteria in a suspension contribute significantly to the latter's bulk capacitance. Also, it should be mentioned that only living cells with intact membranes contribute to bulk capacitance. A cells death is accompanied by loss of membrane potential and electrical polarization [13].

Measuring the bulk capacitance is not straightforward since the electrical behavior of aqueous solutions containing polarizable species, like cells and proteins, is extremely complex. Such systems can be modeled electrically by the circuit shown in Fig. 3 (a) [6]. In this equation, charge stored at the electrode-solution interfaces is accounted for by the two electrode interface capacitances (Ce) and charge stored by elements dispersed in the interior, by "bulk capacitance" (Cb). Measuring changes in Cb is challenging since Ce is roughly 1000 times larger. The key innovation [18] is a method that enables the changes

in Cb to be detected, despite the “screening effect” of charges at the electrode-effective bulk resistance (Rb) of the suspension. This increases the impedance of the bulk (RbCb) to be comparable to that of the interface at realizable frequencies allowing an appreciable voltage drop over the bulk-suspension. Therefore, the charge-storage in the bacteria contributes significantly to the measured impedance. The long narrow channel causes more of the electrical “lines of force” to interact with the suspended bacteria. The measured impedance (Z) at 500 frequencies (w) between 1 kHz and 100 MHz is fit to the equation, and Cb is estimated (along with Rb, Re, and Ce).

The application of this technology has been demonstrated for food quality assessment [18] and blood culture [9]. For blood culture, the “times to detection” (TTDs) were often days shorter than the current market leader. The threshold concentrations of detection are 10^3 - 10^4 CFU/mL, while compared to current technologies 10^8 CFU/ml [8].

Further, since single cells lose their membrane potential when they die and no charge accumulates at the membrane in absence of potential, m-EIS is able to observe cell death in real time. This insight has been previously used to determine minimum inhibitory concentrations (MICs) for multiple bacteria-antibiotic pairs [9]. Besides being rapid, taking 4 hours for *E.coli*, *S. aureus* and *Pseudomonas* detection, and accurate, correct MIC values were obtained for well-characterized strains; it is able to distinguish between bacteriostatic effects, in which the bacteria can no longer replicate, from bactericidal, which will kill the bacteria cell. Moreover, cell death can be observed using m-EIs for initial loads

at or above the “threshold” concentrations of around 10^3 CFU/ml. This was shown for *E.coli* and *Pseudomonas*. Not only does the m-EIS method require a lower threshold concentration, but it also works with small volumes of sample (~10 µL). It thus requires very small number of cells (<100 CFUs) to work. Further (as will be demonstrated in this work), it is unaffected by the presence of inert material like MNPs, even if they happen to be charged or polarizable. The presence of inert species merely contributes to the background, and since m-EIS looks for a change in bulk capacitance from the baseline (time t = 0) value brought about either by cell proliferation or cell death to determine the effect of the candidate drug on the mycobacterial cells, they do not affect the interpretation of the data as long as their number remains constant. Due to a combination of needing low cell numbers to work, and an ability to work in the presence of MNPs, and the potential for relatively easy parallelization, m-EIS is ideally suited to run multiple experiments in parallel and evaluate the MIC of the isolated strain against multiple concentrations of the various antibiotics.

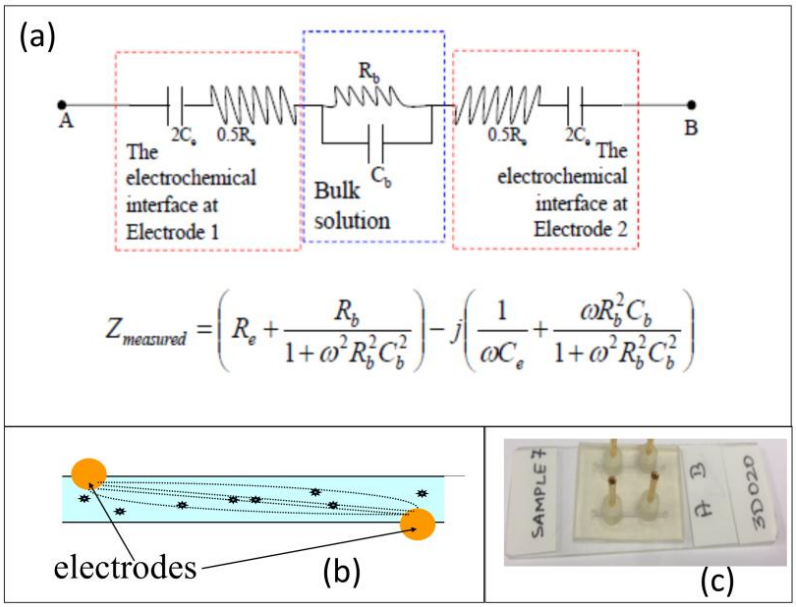


Figure 5. Schematic and Electrical Circuit Model Representation of Microchannel

(a) Electrical Model of an aqueous suspension in contact with metal electrodes. The equation relates the real (in-phase) and imaginary (out-of-phase) components of the measured impedance (Z) and how they vary as a function of frequency and model parameters (R_e, C_e, R_b and C_b) (b) schematic, and (c) picture of microfluidic cassette.

2.7.2 Electrical data collection

Aliquots (~50 µL) taken from the liquid culture and introduced into a microfluidic cassette containing channels with a 1mm x 1mm cross section. These cassettes consist of a glass-slide base, and a 3D printed top with engraved channels affixed using an adhesive (Epo-Tek 301™). The 3D printed part was fabricated at the Mizzou 3D Printing Lab and had electrodes asymmetrically placed 10mm apart, as shown in Figure 5. It thus allows the user to assay a fluid volume of ~10 µL (~10mm x 1mm x 1mm). The electrodes shown were connected to an impedance analyzer (Agilent/Keysight 4294A), and impedance measurements [Resistance (R) and Reactance (X)] were recorded at 128 frequencies (w)

between 1 kHz and 100 MHz. This raw data [R(w) and X(w)] was analyzed offline using ZView™ software to yield an estimate of the suspension's bulk capacitance (Cb). To study the effect of candidate drugs on the cells, such scans were conducted on the same sample multiple times over the duration of the study. The time interval between the reading was ~30 min for *E.coli* and ~40 min for *S.aureus*. At each time-point, 5 independent aliquots were taken from the liquid culture suspension. After loading each aliquot (~50 µL) into cassette, electrical measurements were taken. Then the channel was cleared twice using sterile TSB media. After the channel was cleared, the channel was loaded again with the next ~50 µL aliquot until 5 readings were completed.

2.7.3 Analysis of the Electrical Data

The data collected by the impedance analyzer is in the form of resistance (R) and reactance (X) values of the bacterial suspension at 128 logarithmically equi-spaced frequencies (w) between 1 KHz and 100 MHz. As described by us previous [17] the R and X vs. ω data is fitted to the equivalent electrical circuit shown in Figure 4 using a commercially available software package (ZView™). The software accepts as an input the Z vs. ω data, and provides an estimate for all the circuit parameters, including the "bulk capacitance" (Our parameter of interest that provides a measure of charges stored in the interior of the suspension (away from the electrodes)). It may be noted that here, (a) the capacitances and resistances at both electrodes are combined into a single capacitance and resistance, respectively, and (b) both the bulk and interfacial capacitances are represented as a constant-phase element (CPE) to account for

the non-ideal nature of the capacitance at cell membranes and electrodes, where charges carried by ions take a finite time to accumulate, unlike an ideal capacitor where the accumulation is instantaneous. The magnitude of the bulk CPE, thus, reflects the amount of charge stored at the membranes of living microorganisms in suspension. An increase in the number of living microorganisms (brought about by cell proliferation) should be reflected as an increase in this value (magnitude of CPE). On the other hand, a decrease in the number of living microorganisms in the suspension (due to the action of the drug present) should lead to a lower bulk capacitance (CPEb - T).

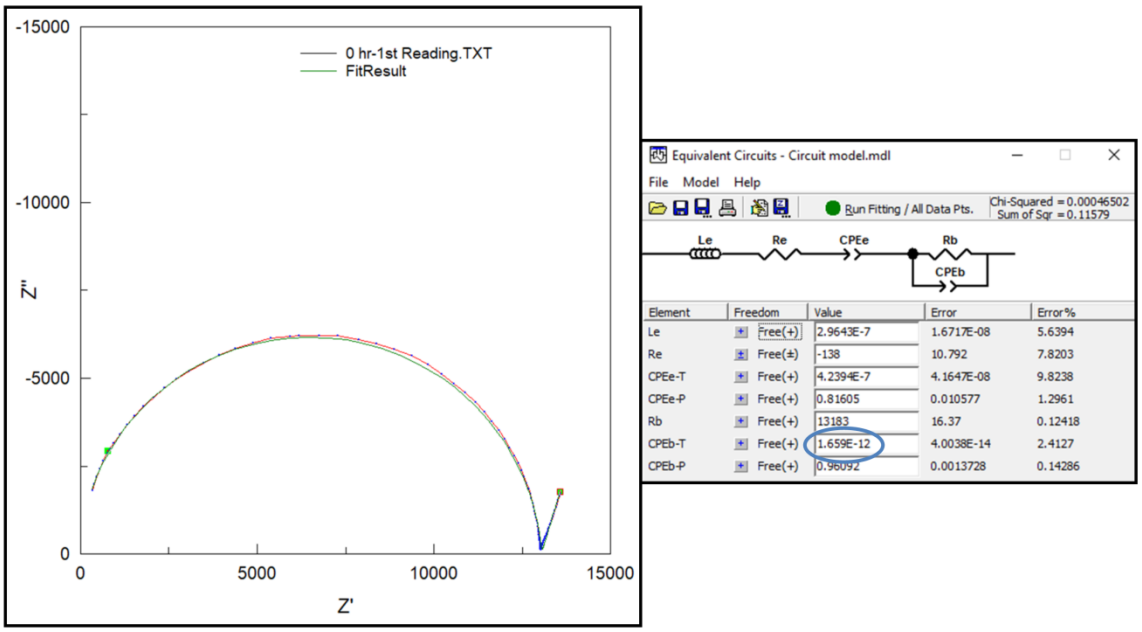


Figure 6. Data Analysis using the program ZVIEW®

The left side shows the graphical fit-line to the Resistance (Z') and Reactance (Z'') which is fit to the circuit of choice (top right). The fit results in an estimate of the bulk capacitance (circled, bottom right).

Chapter 3. Results and discussion:

3.1 - CFU counts from positive blood culture samples and Extraction using MNPs:

As discussed earlier and illustrated in figure 2, our method involves extraction of the microorganisms from the positive patient blood culture broth prior to antibiotic susceptibility testing using the novel m-EIS method. Prior to extraction, the microbial concentrations from positive blood culture samples were determined by plating the positive blood culture sample (after appropriate dilutions) and calculating the colony forming units (CFU) per ml. The table 2 shows the average concentration of microorganisms at the time the samples were deemed positive for the presence of microorganisms in the blood culture broth. Even though the Time to positivity (TTPs) were different for different bacteria and yeast strains, the final concentration at TTP were found to be around 1×10^7 to 10^8.

These positive blood culture broths were then used to determine the extraction efficiencies utilizing the commercial MNPs. The extraction efficiencies were calculated as the ratio of estimated load (in log) after extraction to that of load before extraction. The results for the various microorganism extractions from positive blood culture broth are shown in figure 7.

As can be seen in the figure 7, the microorganisms maintain viability when going through the capture process using MNPs. The extraction efficiencies are >80% for multiple organisms tested. The organisms used include gram positive bacteria, gram negative bacteria and yeast, all of which demonstrate high extraction efficiencies indicating the versatile nature of the MNPs and the protocol employed for extraction. Once extracted, the microorganisms and MNPs

are resuspended in Mueller Hinton broth (MHB), and the subsequent behavior of microorganisms is expected to depend solely on their nature since little to no trace remains of the original biological fluid they were present in. The only concern that one needs to then address is whether the presence of MNPs affect the measured parameter of interest, viz. the Minimum Inhibitory Concentration (MIC).This has been addressed in the next section.

Organism	Colony Count in CFU/ml
E. coli	3.18E+07
P.aeruginosa	7.90E+07
E.faecalis	6.84E+09
S.aureus	3.13E+08
K.pneumonia	3.13E+08

Table 2. Blood Culture Extraction at Time to Positivity (TTP)

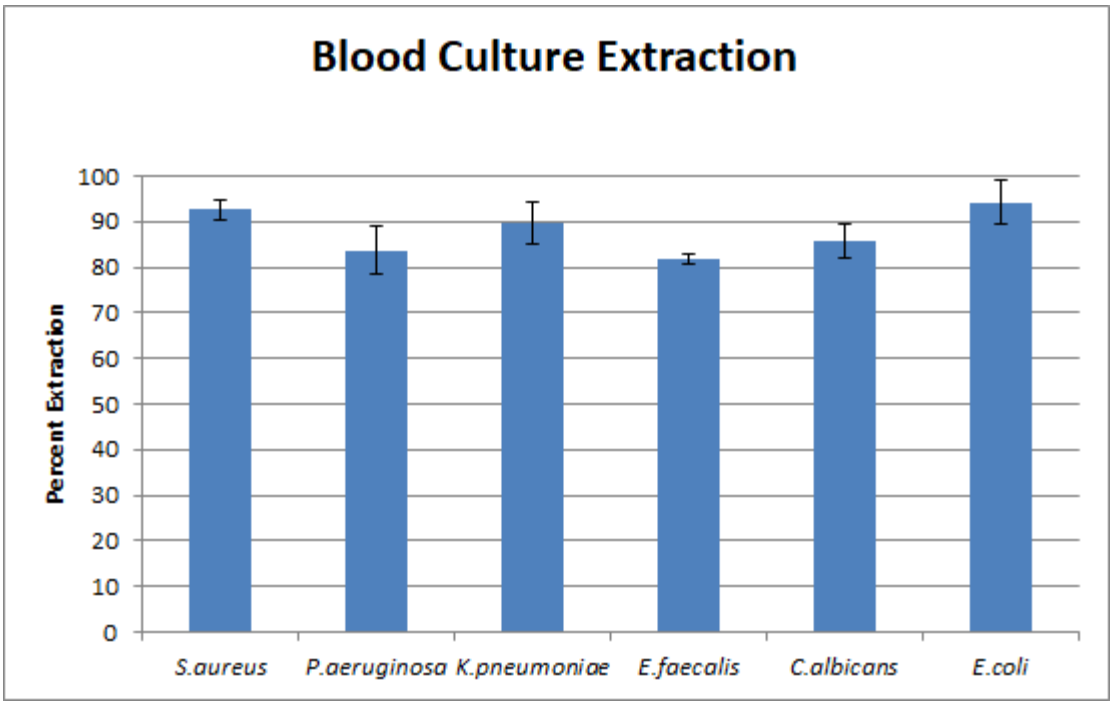
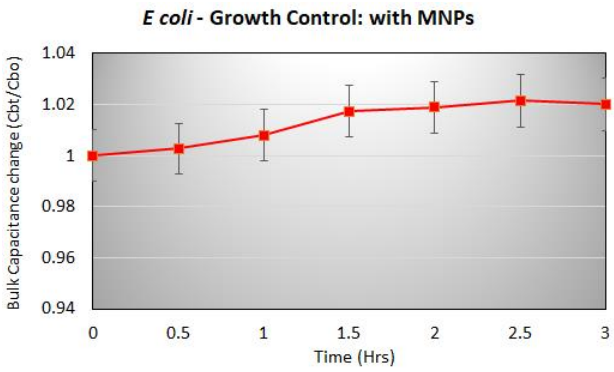
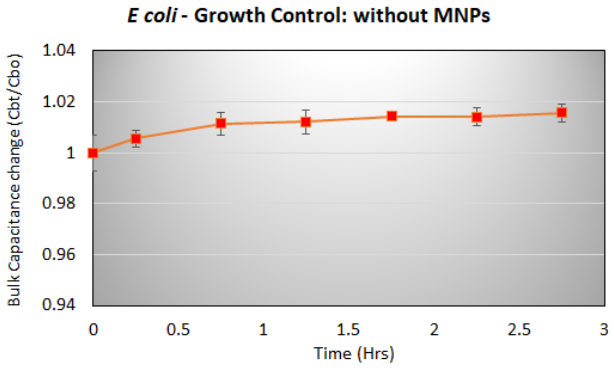
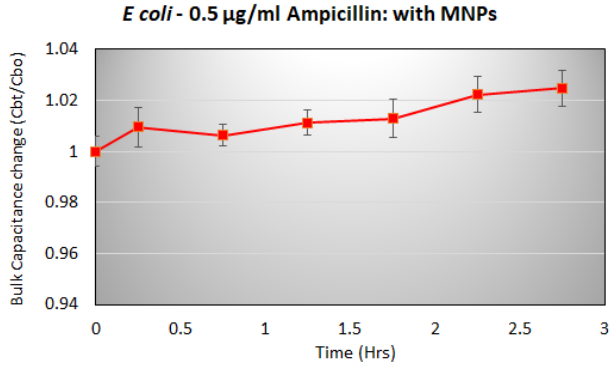
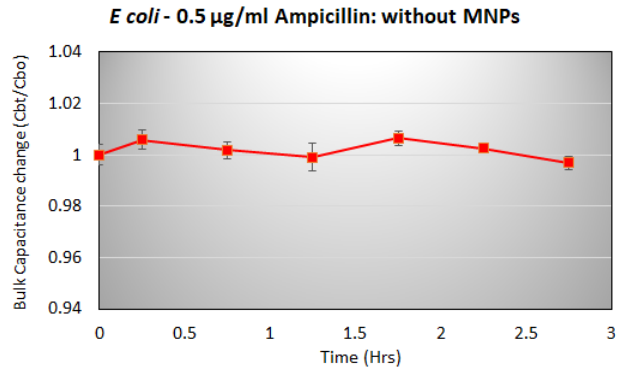


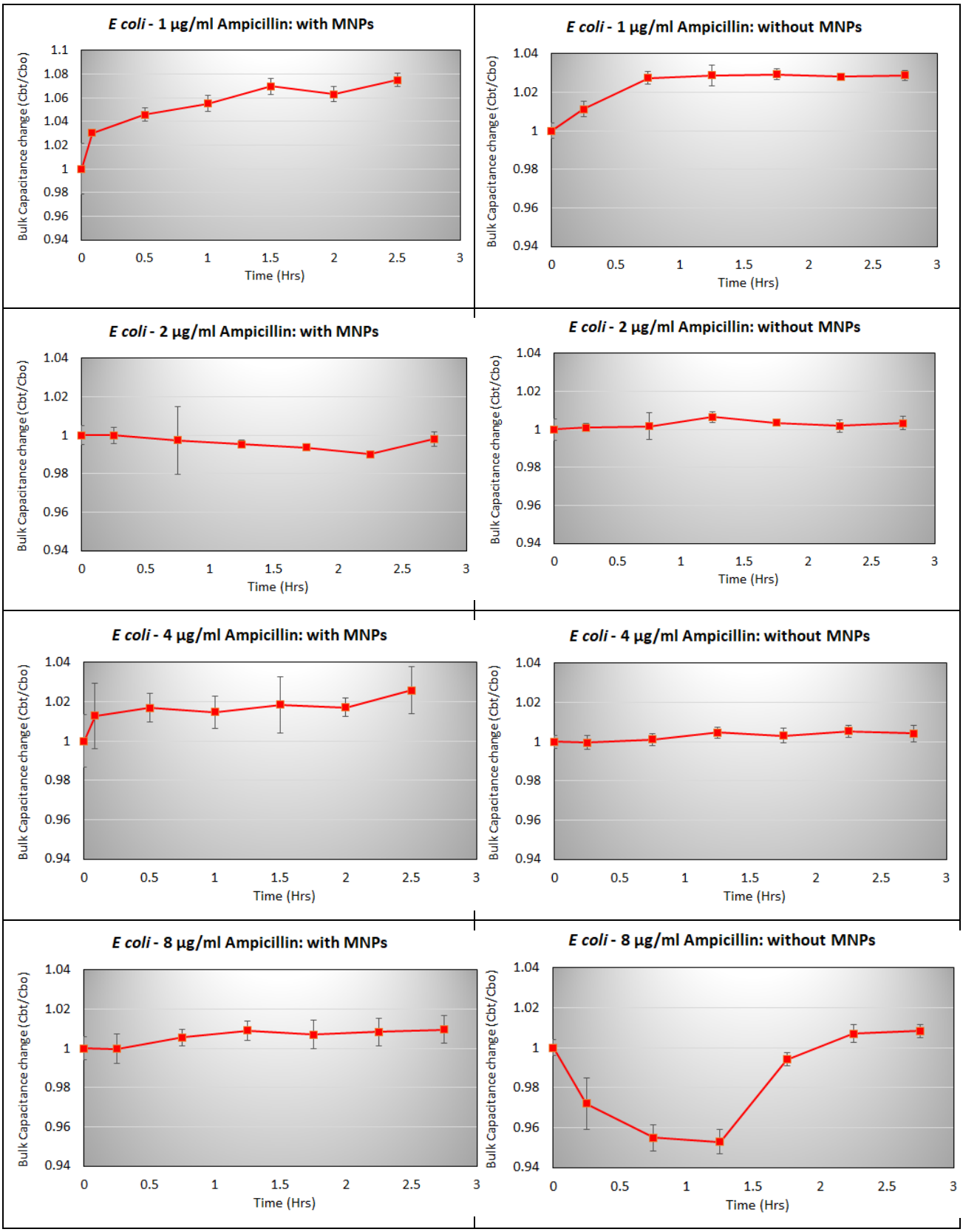
Figure 7. MNP extraction of microorganisms

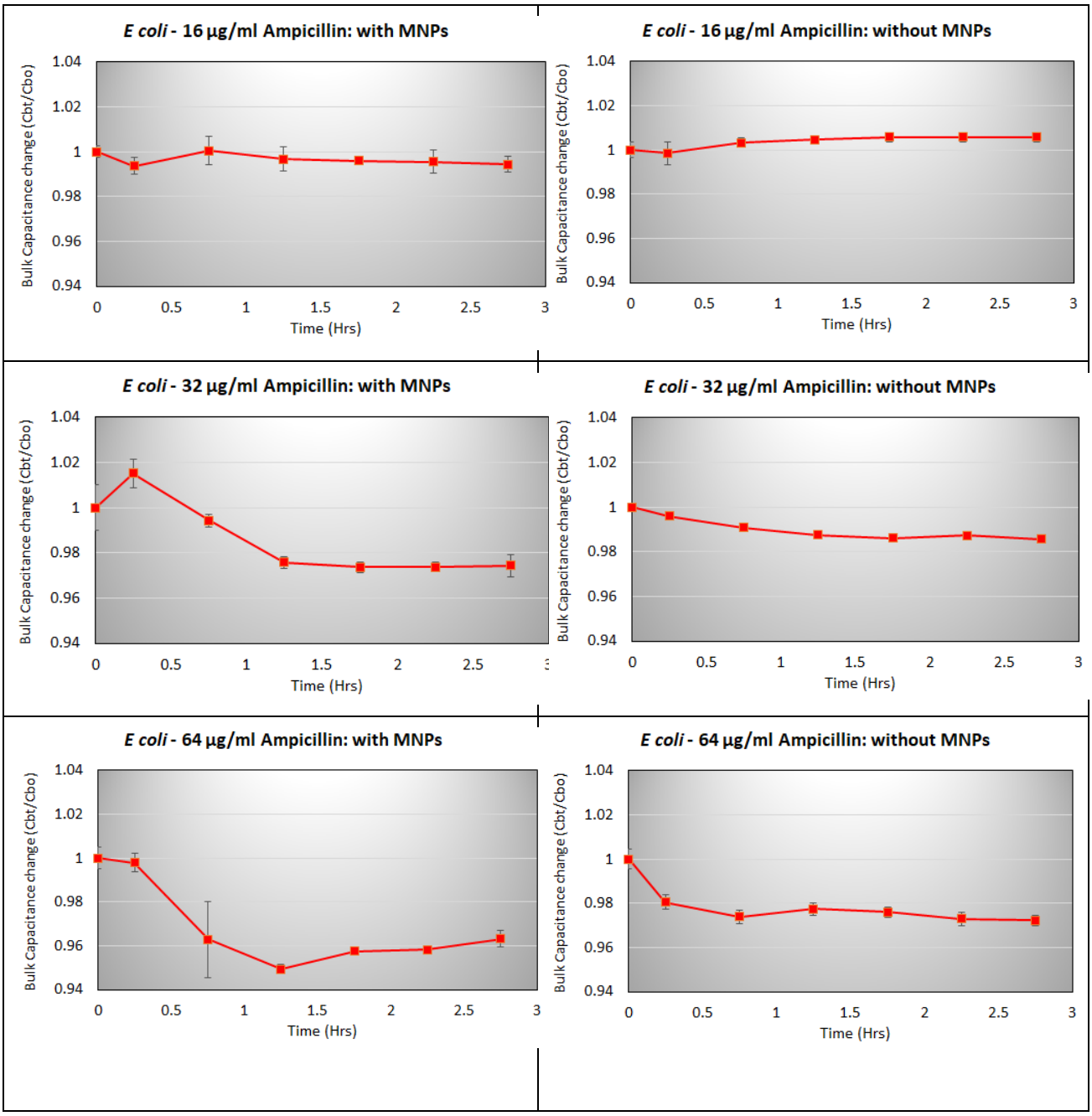
3.2. m-EIS:

As explained earlier, m-EIS experiments were conducted wherein candidate drugs at different concentrations were allowed to act on samples mimicking those that we expect to obtain after the sample-preparation process (in terms of loads of living mycobacteria and the MNPs present), and the measured bulk capacitance tracked over a period of time (3 to 4 hours for *E.coli* and *S.aureus*). To verify that MNPs do not affect either the behavior of the cells (with respect to their susceptibility to the drugs tested), or the m-EIS method to monitor the said growth or death, other experiments are run in parallel where conditions are similar to the first set of experiments, but where MNPs are not present. Results (values of bulk capacitance, scaled to the time t = 0 “baseline” value) are shown in Figure 8 and Figure 9 for *E. coli* and *S. aureus* respectively. Table 3 and Table 4 shows the inference one can draw regarding growth/stasis of the

bacteria based on how these measured values of bulk capacitance change over time. The tables show the estimates of the slope of the straight line fitted to the average of the measured bulk capacitance vs. time data with 95% confidence interval (CI). If upper and lower bounds of the confidence interval lies entirely in the positive range, bacteria in the sample are deemed to be proliferating; if the CI is entirely within negative range, bacteria are deemed to be dying, and if the CI overlaps zero, bacteria are deemed to be static under the conditions tested.

E. coli	
With MNPs	No MNPs
	
	


E coli - 1 µg/ml Ampicillin: with MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 1 µg/ml Ampicillin: without MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 2 µg/ml Ampicillin: with MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 2 µg/ml Ampicillin: without MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 4 µg/ml Ampicillin: with MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 4 µg/ml Ampicillin: without MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 8 µg/ml Ampicillin: with MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
E coli - 8 µg/ml Ampicillin: without MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)


E coli - 16 µg/ml Ampicillin: with MNPs
E coli - 16 µg/ml Ampicillin: without MNPs
E coli - 32 µg/ml Ampicillin: with MNPs
E coli - 32 µg/ml Ampicillin: without MNPs
E coli - 64 µg/ml Ampicillin: with MNPs
E coli - 64 µg/ml Ampicillin: without MNPs
Bulk Capacitance change (Cbt/Cbo)
Time (Hrs)
1.04
1.02
1
0.98
0.96
0.94
0
0.5
1
1.5
2
2.5
3

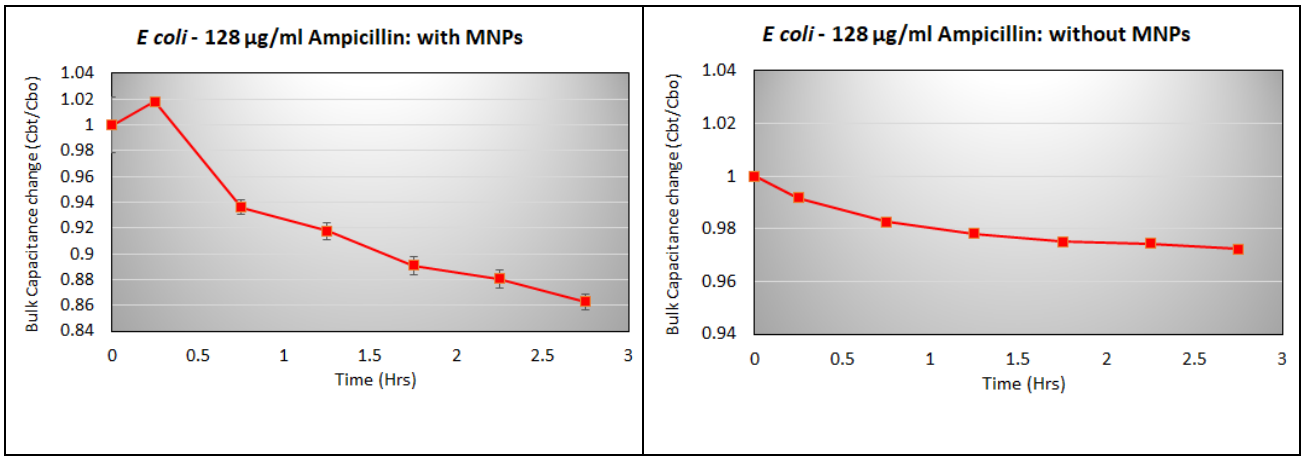
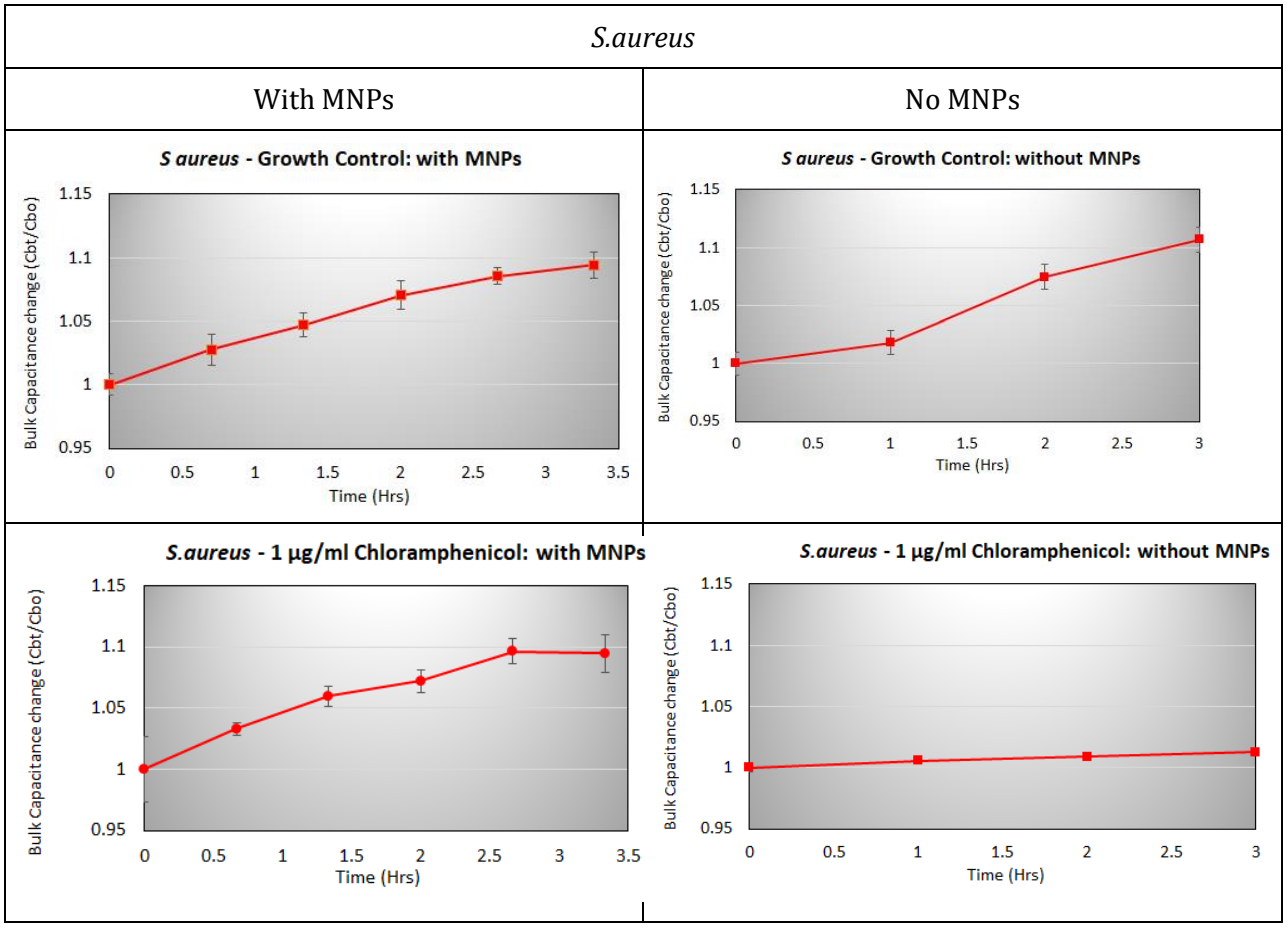
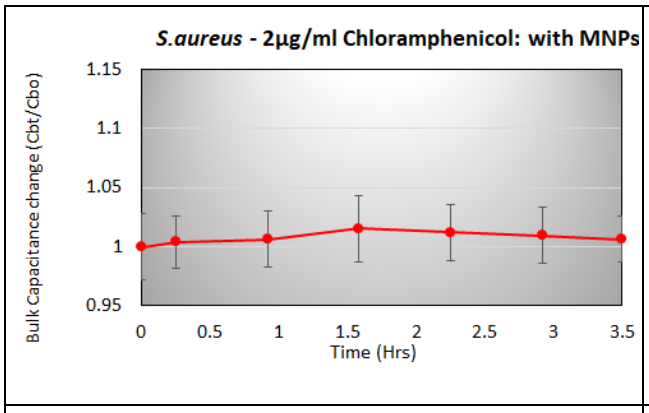
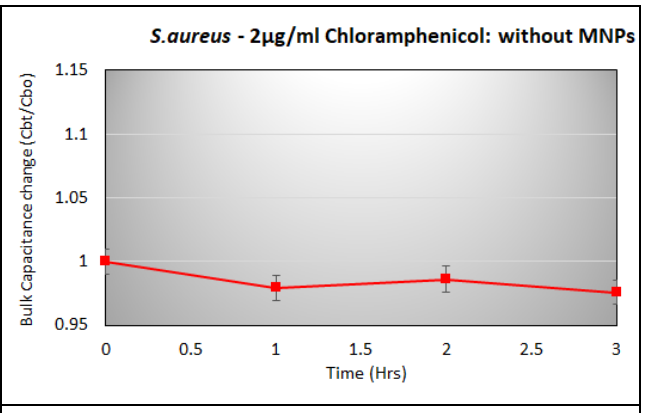


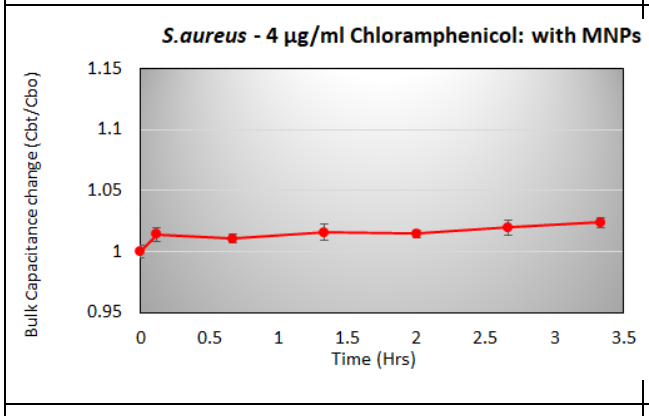
Figure 8. Bulk capacitance Changes over time for *E.coli*

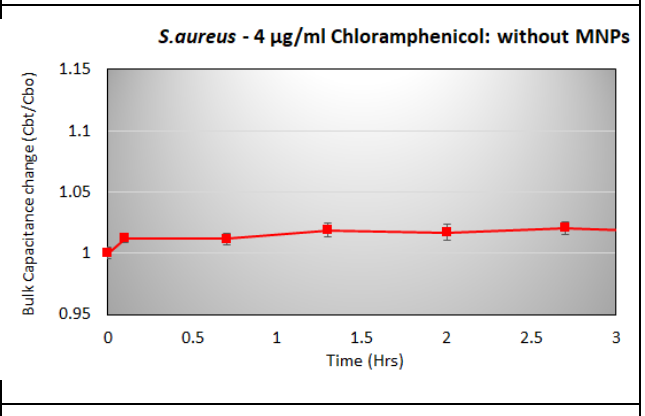
Change in the value of the measured bulk capacitance (Cb) as a function of time for E. coli cultures with and without Magnetic Nano-particles (MNPs) when exposed to cidal and static antibiotics. All changes are normalized to the baseline (time t = 0) value. (n=5 at each data point)

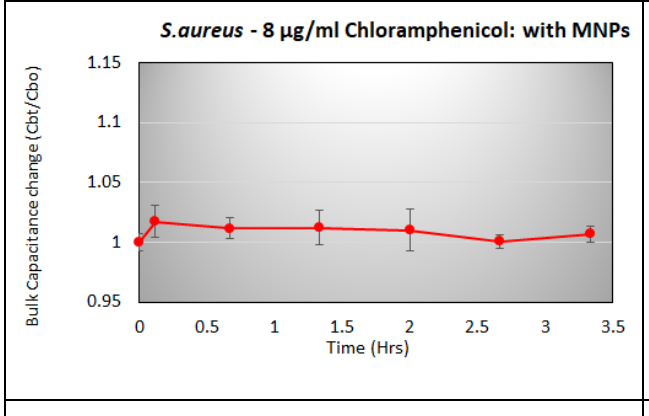


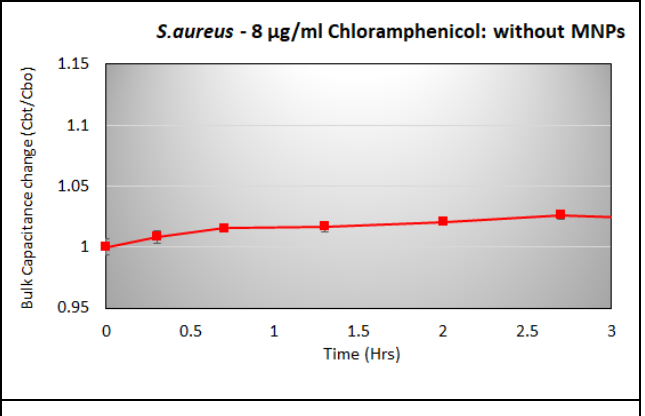

S.aureus - 2µg/ml Chloramphenicol: with MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
3.5
Time (Hrs)

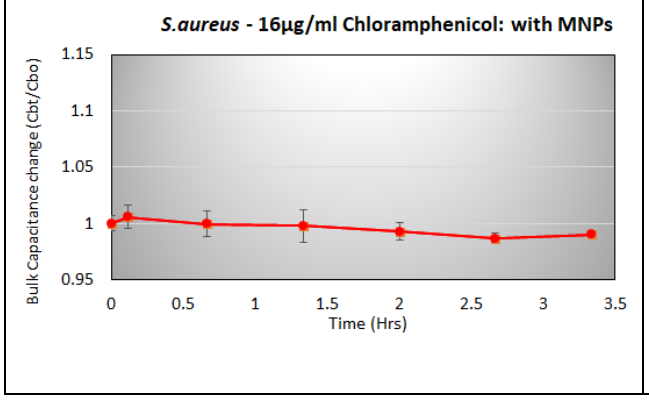

S.aureus - 2µg/ml Chloramphenicol: without MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
Time (Hrs)

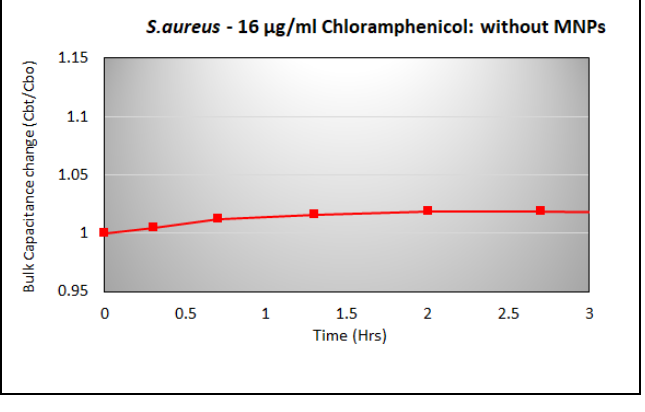

S.aureus - 4 µg/ml Chloramphenicol: with MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
3.5
Time (Hrs)


S.aureus - 4 µg/ml Chloramphenicol: without MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
Time (Hrs)


S.aureus - 8 µg/ml Chloramphenicol: with MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
3.5
Time (Hrs)


S.aureus - 8 µg/ml Chloramphenicol: without MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
Time (Hrs)


S.aureus - 16µg/ml Chloramphenicol: with MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
3.5
Time (Hrs)


S.aureus - 16 µg/ml Chloramphenicol: without MNPs
Bulk Capacitance change (Cbt/Cbo)
1.15
1.1
1.05
1
0.95
0
0.5
1
1.5
2
2.5
3
Time (Hrs)

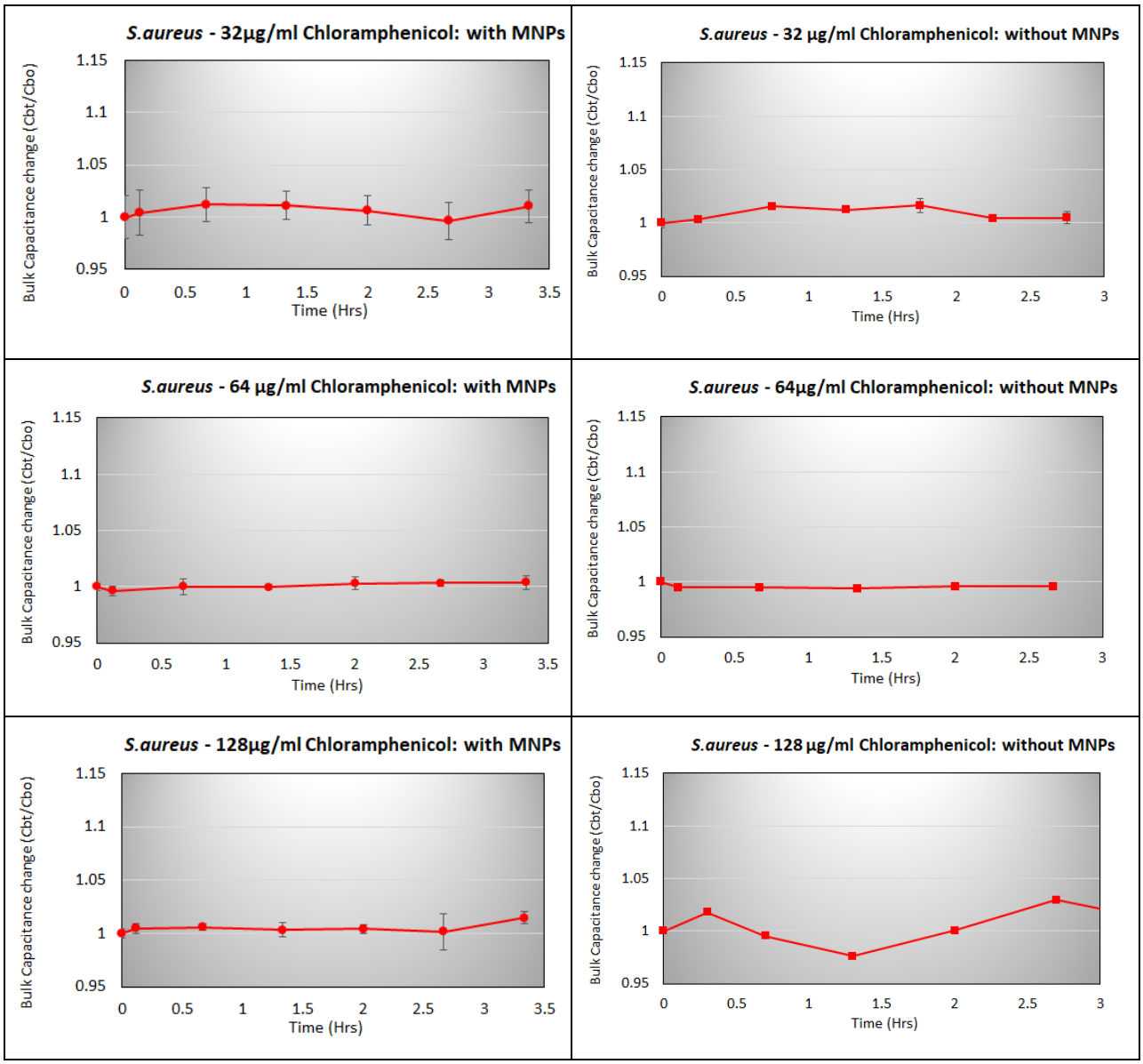


Figure 9. Bulk capacitance Changes over time for *S.aureus*

Change in the value of the measured bulk capacitance (Cb) as a function of time for S. aureus cultures with and without Magnetic Nano-particles (MNPs) when exposed to cidal and static antibiotics. All changes are normalized to the baseline (time t = 0) value. (n=5 at each data point)

To ensure unbiased analysis of the data, the following statistical approach is adopted to determine whether one is observing growth, death or stasis in a given experiment: The slope of the C_b vs. time for the first 3 hours is then

calculated, along with the 90% confidence interval of its value. *In other words, we use the data to obtain an estimate of the rate of increase or decrease of the C_b value*. If the confidence interval (CI) of the slope is entirely positive, then one can infer that the bacteria are growing for the given amount of antibiotic. Conversely, a confidence interval of the slope that is entirely negative indicates that the bacteria are being killed. A case where the confidence interval of the slope overlaps zero (upper bound is >=0, while lower bound is <=0) indicates that the action of the antibiotic is static. By definition, the Minimum Inhibitory Concentration (MIC) is the minimum concentration of the antibiotic which causes either stasis or death, and hence in our case, it is the lowest concentration of antibiotic for which the confidence interval overlaps zero (or is entirely negative).

In Figures 10, we plot the slopes recorded (our estimates of the rates of increase/ decrease) along with the *confidence intervals (error bars)* for *E. coli ATCC 25922* against various concentrations of ampicillin, and *S. aureus ATCC 29213* against various concentrations of chloramphenicol, respectively. As seen, not only do we observe the expected behavior (cidal vs. static)for both gram-positive and gram-negative bacteria, our calculated MIC (red arrow) is the same with and without MNPs present, and within the expected range of MICs for that particular strain [10] (shaded zone). In other words, the MIC values we obtain using our method are “correct”, despite the presence of potentially interfering agents (MNPs).

E.coli						
Sample Type	**Antibiotic**	**Concentration of Antibiotic (µg/mL)**	**Estimate of Slope**	**95% CI Lower Bound**	**95% CI Upper Bound**	**Inference**
With MNP	Growth Control	0	0.00738	0.00261	0.0121	Growth
	Ampicillin	0.5	0.038	0.038	0.038	Growth
		1	0.03	0.017	0.043	Growth
		2	-0.011	-0.023	0.001	Static
		4	0.003	-0.001	0.007	Static
		8	0.002	-0.002	0.006	Static
		16	0.000	-0.002	0.003	Static
		32	-0.034	-0.060	-0.008	Death
		64	-0.032	-0.055	-0.009	Death
		128	-0.049	-0.058	-0.041	Death
Without MNP	Growth Control	0	0.00342	0.00113	0.0057	Growth
	Ampicillin	0.5	0.011	0.0054	0.0162	Growth
		1	0.016	0.004	0.028	Growth
		2	0.003	-0.001	0.0077	Static
		4	0.001	-0.00076	0.0029	Static
		8	-0.013	-0.046	0.0194	Static
		16	0.004	-0.001	0.0092	Static
		32	-0.004	-0.006	-0.001	Death
		64	-0.009	-0.016	-0.003	Death
		128	-0.024	-0.034	-0.014	Death

Table 3

Estimates of slope of the straight line fitted to data consisting of measured bulk capacitance value (C_b) (dependent variable) as a function of time (t) (independent variable) for various conditions, along with the lower and upper bounds of the 95% confidence interval for E. coli.

S.aureus						
Sample Type	**Antibiotic**	**Concentration of Antibiotic (µg/mL)**	**Estimate of Slope**	**95% CI Lower Bound**	**95% CI Upper Bound**	**Inference**
With MNP	Growth Control	0	0.02603	0.0804	0.0340	Growth
	Chloramphenicol	1	0.045	0.026	0.065	Growth
		2	0.003	-0.003	0.009	Static
		4	0.003	-0.005	0.012	Static
		8	-0.001	-0.008	0.005	Static
		16	0.000	-0.005	0.005	Static
		32	0.002	-0.005	0.009	Static
		64	-0.001	-0.006	0.005	Static
		128	.0.001	-0.005	0.006	Static
Without MNP	Growth Control	0	0.00393	0.02365	0.0574	Growth
	Chloramphenicol	1	0.015	0.007	0.023	Growth
		2	-0.007	-0.029	0.015	Static
		4	0.003	-0.0003	0.005	Static
		8	0.002	0.00	0.00	Static
		16	0.002	0.00	0.00	Static
		32	-0.003	-0.006	0.00	Static
		64	0.000	-0.005	0.005	Static
		128	-0.001	-0.008	0.007	Static

Table 4

Estimates of slope of the straight line fitted to data consisting of measured bulk capacitance value (Cb) (dependent variable) as a function of time (t) (independent variable) for various conditions, along with the lower and upper bounds of the 95% confidence interval for S. aureus.

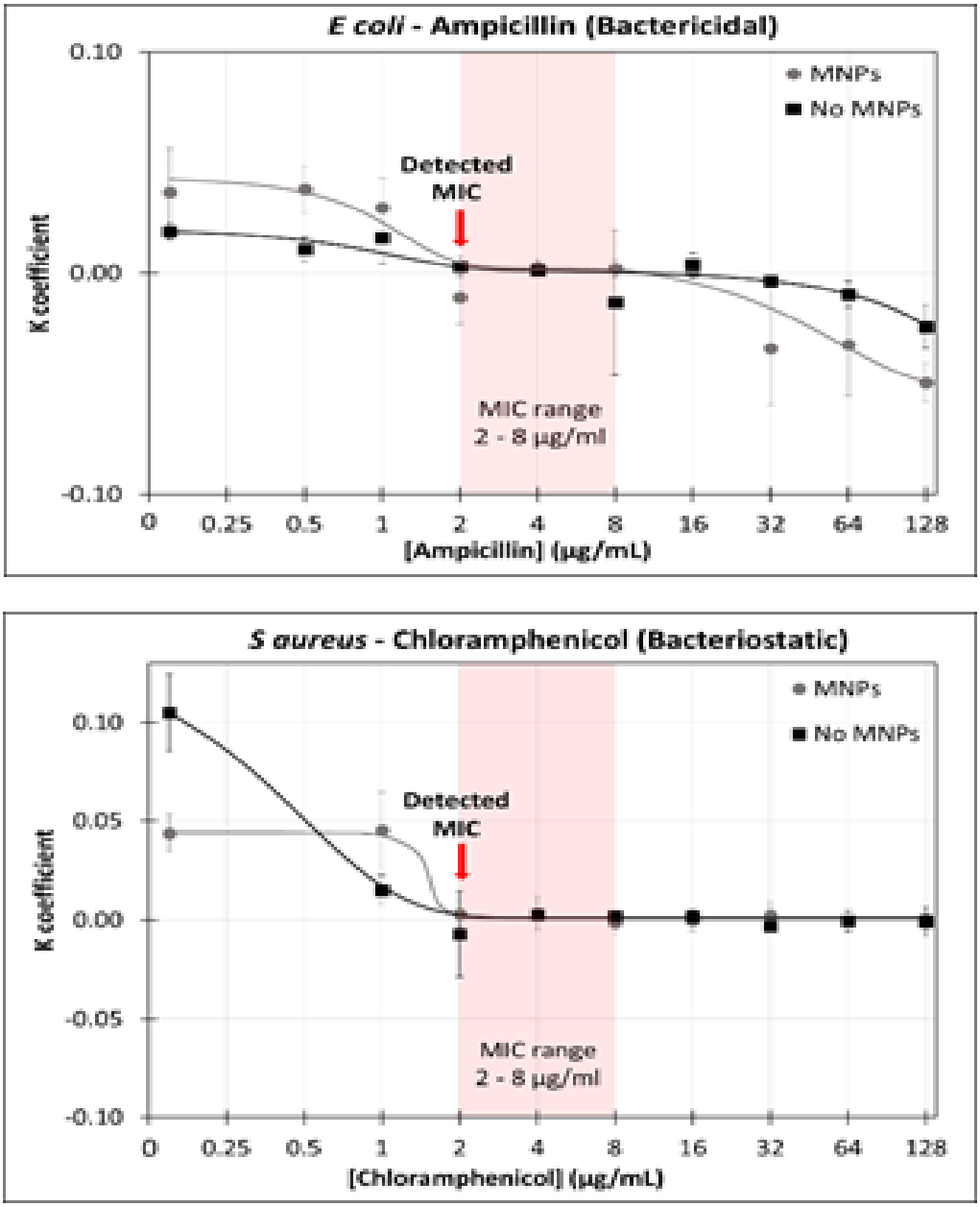


Figure 10. Determination of MIC

Estimates of the rate of increase/decrease in the value of the measured bulk capacitance (C_b) as a function of antibiotic concentration. Error bars represent 90% Confidence Intervals of the estimate. The shaded region represents the expected MIC range for the given strain against the given antibiotic. MIC (minimum Inhibitory Concentration) is the smallest concentration at which the confidence interval of the estimated rate either includes zero (no growth, statistically) or is completely in the negative (death). As seen, the measured MIC falls within the expected range for all cases (standard suspensions and suspensions with MNPs). Also, bactericidal and bacteriostatic effect can be distinguished.

Chapter 4. Conclusion and Future Work:

Through this work, we demonstrate proof of principle that (a): The microbial load at the time to positivity (TTP) for a blood culture (in CFU/ml) falls within a relatively narrow range (~10^7 to 10^8 CFU/ml), irrespective of the type of organism; (b) various types of microorganisms, spanning that variety that causes blood stream infections (gram positive bacteria, gram negative bacteria, and yeasts), can be recovered from positive blood culture broths using commercially available MNPs with high efficiency (>85%), and (c) that the efficacy of candidate antibiotics on bacteria thus isolated (in suspensions containing MNPs) could be tested in real-time using m-EIS.

From (a) and (b) above, it follows that we can hence follow standardized dilution protocols without the need to know what the ID of the organism is, and obtain suspensions containing somewhere between 10^5 to 10^6 CFU/ml of pathogen cells. Further, as can be seen from our m-EIS experiments (figures 8, 9, and 10, and Tables 3 and 4) the MNPs do not seem to affect the behavior of the cells they come into contact with in a manner detrimental to our purpose (The pathogens not only remain alive, but continue to display the “correct” minimum inhibitory concentrations). Also our m-EIS results (both with and without MNPs) are consistent with our expectations in the sense that (a) electrical signatures of growth (increase in bulk capacitance) are observed for controls (no drugs), for drugs known to be bacteriostatic and bactericidal drugs present at concentrations below MIC, (b) electrical signatures of death (decrease in bulk capacitance) are seen for cidal drugs at concentration at and/or above MIC, and (c) electrical signatures corresponding to bacterial stasis (no significant change in bulk

capacitance over time) can be seen for bacteriostatic drugs at and above the MIC. Moreover, similar samples with and without MNPs display similar behavior, thereby laying to rest our apprehensions regarding the potential interference with either the bacterial behavior or the electrical readings.

One major limitation of the current study is that the two parts of the study (isolation using MNPs and m-EIS) were conducted independently, although care was taken to ensure that the composition of the suspensions subjected to m-EIS was similar to those obtained at the end of the isolation protocol. Another limitation was that aliquots from a larger culture (~ 10ml) were introduced into the microchannel by hand at every point in time.

Future work will be directed towards developing a microfludic cassette so that the process as proposed in Figure 2 can be realized. In other words, the microfludic cassette will contain multiple channels, each with known amounts of antibiotic pre-loaded in dried / lyophilized form, and capable of being sealed after fluid is introduced (via septa, luer locks or other mechanisms) to eliminate evaporative losses. This will enable the user to take cells isolated using MNPs and load them directly into the channels, where their response to the antibiotics can be electronically monitored. Monitoring multiple channels simultaneously will also require additional development of the circuitry, which is currently capable on monitoring only one channel at a time.

5. References

1. Wang, X., et al., *Bead capture increases the sensitivity of sputum microscopy for the diagnosis of tuberculosis in Beijing, China.* 2013. **107**(11): p. 741-743.
2. Jin, Y., et al., *Biofilm-forming ability of Candida albicans is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection.* 2003. **41**(7): p. 2961-2967.
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