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Detection by death: A rapid way to detect viable slow-growing microorganisms achieved using microchannel Electrical Impedance Spectroscopy (m-EIS)

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Based on the insight that only living organisms can be killed (and that killing can proceed much faster than cell-growth), we present an approach for the detection of viable microorganisms that is much faster than currently used culture-based methods. We do so by using microchannel Electrical Impedance Spectroscopy (m-EIS) for real-time detection of cell-death on exposure to a killing-agent.

m-EIS relies on the fact that when living-cells with non-zero membrane potentials are exposed to high-frequency AC-field, induced-charges accumulate at the membrane-interface. Cell-death is accompanied by a loss of membrane-potential, and hence charge-storage (capacitance).

A proof-of-principle for a clinical-application (detection of living mycobacteria in sputum) is demonstrated. *Mycobacterium smegmatis* (doubling-time ~3 hours) and *Mycobacterium bovis BCG* (doubling-time ~20 hours) in artificial-sputum are both detected in <3 hours when exposed to amikacin. Times-to-detection (TTDs) are ~12 hours and ~84 hours (3 1/2 days), respectively for culture-based detection using current technologies (BD-MGIT-960™) for samples containing similar loads of *M. smegmatis* and *M. bovis BCG*.

***Keywords*: Electrical Impedance Spectroscopy; Mycobacteria; Time-to-Detection; Cell-Death; MGIT 960 System.**

INNOVATION OF THE TECHNOLOGY

In trying to detect viable microorganisms, automated culture-based systems (BACTEC, BacT/Alert, MGIT, etc.) ask "do they metabolize and/or proliferate?", and try to detect signatures of bacterial metabolism/growth (changes in pH, solution-conductivity, O_2/CO_2 levels, etc.). In contrast, we ask, "can they be killed"? Since only living entities can be killed, an answer in the affirmative should confirm the presence of live entities (microorganisms). Doing so makes our time-to-detection (TTD) dependent not on the metabolic-rate of the microorganisms, but on how fast they are killed.

We detect death by measuring a parameter (charge stored at the membranes of cells with a non-zero membrane-potential under an AC-field) that falls when microorganisms are killed (membrane-potential goes to zero). We demonstrate its use for mycobacteria, which have long doubling-times (~24 hours), and for which current culture-based systems take days/weeks to detect. Since they can be killed quickly (in minutes/hours), we can do so in 3 hours or less.

INTRODUCTION

Motivation

In many real-world situations, one seeks to detect ***viable*** (live) microorganisms present. The material under investigation, often, is known or expected to contain non-viable microbial cells present. In many cases, it is also of utmost importance to obtain this information as quickly as possible. Examples include (but are not limited to) (a) the need to detect the presence of viable bacteria and/or yeasts in blood for patients suspected of having an active bloodstream infection (septicemia), where it is possible that other non-viable bacteria are also present[1,2], (b) the need to check for the presence of coliforms and other bacteria in food or water samples after they have been subjected to procedures such as pasteurization or disinfection[3], and (c) the need to detect viable cells of *Mycobacterium tuberculosis* (the organism that causes Tuberculosis (TB)) in the sputum of patients suspected of having an active infection (given that dormant *M. tuberculosis* cells may be present in cases of "latent" TB[4], or previous treatment may have left behind some dead cells of *M. tuberculosis*)[5].

In such cases, the need to prevent false positives due to the presence of dead cells excludes some technologies such as DNA based methods like PCR and antibody based approaches like ELISA as viable options[2]. Given the above limitation (presence of dead cells) and added constraints brought about by the desire to contain costs, and make the detection automated and not dependent on human judgement, automated culture-based systems currently serve as the work-horses of the microbiology laboratory for these types of applications. Some commonly encountered automated culture based detection systems include blood culture systems like the BACTEC™ from Becton-Dickinson (BD), the BacT/Alert™ from Biomerieux and Versa-TREK from Thermo-Scientific, specialized culture systems for mycobacteria like the Mycobacteria Growth Indicator Tube

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(MGIT) from BD, and Trek-ESP from Thermo-Scientific, and products like RABIT, BacTrac, Malthus 2000 that are used primarily for food and water testing.

In general, the protocol followed in automated culture-based systems require the user to add an aliquot of the sample of interest (blood, sputum, food, etc.) into a bottle containing nutrient broth conducive to the target microorganisms. These microorganisms, if present, metabolize compounds such as sugars and proteins/peptides present in the nutrient broth and grow in number via reproduction. As they do so, they change the properties of the medium such as O_2/CO_2 levels, pH, electrical conductivity, etc. While the specific membrane property monitored differs from instrument to instrument, all automated culture based systems monitor these properties continually (every few minutes at the longest) and generate a notification for the user when the property has changed significantly from the baseline (time $t = 0$) value. Thus, they not only provide for a "load and forget" user experience but also are reliable due to their rather straightforward detection methods and low-cost due to their not needing expensive specialized chemicals. The main drawback of these instruments is the long time that they need to detect the presence of microorganisms. The time to detection (TTD) can range from 1–5 days for blood culture[6,7] to up to 6 weeks for tuberculosis[8]. Two factors (low initial load and a long doubling time of the microorganisms present) adversely affect TTD. Typically, due to the low absolute rate of metabolism of a small bacterial cell (it is estimated that even a fast-growing bacteria like *E. coli* consumes only 2×10^{14} moles of O_2/hr[9] and hence has correspondingly low rates of CO_2/acid production), the bacterial load in the culture tubes being monitored must rise to ~10^8 CFU/ml in instruments like the BACTEC before they are detected[10].

Other approaches have been tried to reduce the TTD in culture-based systems. Gomez-Sjoberg and co-workers[11] concentrated the bacteria present in relatively larger volumes into a small volume using dielectrophoresis (DEP), and thus raised the effective starting concentration of the bacteria before trying to detect changes in solution conductivity brought about by the bacterial metabolism. By doing so, they obtained times to detection (TTDs) of ~2 hours for suspensions of *Listeria monocytogenes* with initial loads of ~10^5 CFU/ml (concentrated using DEP to effective initial loads of ~10^7 CFU/ml) as opposed to ~ 8 hours to detect samples with similar loads without pre-concentration. It may be noted that in this case, the "threshold" concentration for the system to flag the sample as positive remains similar to that of the current instruments on the market. The 4-fold reduction in the obtained TTD is due to pre-concentration alone. On the other hand, in our earlier work[7,9,12], we developed a method that we call microchannel Electrical Impedance Spectroscopy (m-EIS), wherein we measure a parameter (charge storage in the interior of a suspension due to the polarization of membranes of living cells, a.k.a "bulk capacitance") that we find to be more sensitive to changes in bacterial load, and using which proliferating bacteria can be detected at threshold concentrations ~10^3 to 10^4 CFU/ml (as opposed to 10^8 CFU/ml in other systems). We thus obtained TTDs of 2 hours for *E. coli* with initial loads of 100 CFU/ml (without the need to resort to any pre-concentration steps)[12].

While these approaches do reduce the long times to detection (TTDs) associated with automated culture-based systems, the TTDs remain unacceptably long for organisms whose metabolism is slow (doubling times are long). A clinically important example of such an organism is *Mycobacterium tuberculosis*, which has a doubling time of ~24 hours[13] (compared to ~20 minutes for *E. coli*[12]). Using systems currently on the market (such as MGIT™), TTDs for clinical samples containing ~1000 CFU/ml can range from ~200 (8.3 days) to ~800 (33.33 days) hours[14]. Even using our m-EIS method, we obtained only a modest (approximately 2×) reduction in TTD for *Mycobacterium bovis BCG* (a closely related biosafety level II organism with a doubling time of ~20 hours[15]). [Our TTD is 60 hours (2½ days) for initial loads of ~1000 CFU/ml, as opposed to 131 hours (~5½ days) taken by MGIT™ for a similar sample].

Thus, the bottom line is that if one tries to detect living bacteria by asking "are they metabolically active?" or "do they grow?" one is limited by the growth/metabolic rate of the organisms (which may be unacceptably slow). On the other hand, cells can be killed at a much faster rate than growing them, and any technique that studies cell death in real time will be able to determine the presence of bacteria in a system much faster. Since only living cells can be killed, such a system will be able to determine the presence of viable bacteria in the system.

Here, we monitor cell death in real-time using microchannel Electrical Impedance Spectroscopy (m-EIS), a novel, patented method[16] distinct from classical "impedance microbiology" approaches[17]. In classical "impedance microbiology" changes to the electrical properties (either solution conductivity[18] or capacitance of the electrode solution interface[19], or a combination of the two[20]) brought about by bacterial metabolism is detected. Viable bacteria in growth phase break down sugars to more conductive species (such as lactate and carbonate) making the solution more conductive. Interfacial capacitance (C_i) changes as the ions in the double-layer are in electrochemical equilibrium with those in bulk. Hence, these methods can only distinguish between growth and no-growth and cannot distinguish between no-growth and cell death (when neither solution conductivity or capacitance changes). However, our method relies on the fact that in the presence of high-frequency AC electric fields, charge accumulates at the membranes of cells across which there exists a potential difference (the membrane potential of living cells)[21]. The charge storages (capacitances) at individual cells contribute to the overall "bulk capacitance" of the suspension (net charge stored in the interior). As the number of living cells increases (due to proliferation), the bulk capacitance increases. Further, as cells die, the membrane potential falls significantly[21], and charge storage under an AC field is absent at the membrane leading to a decrease in the bulk capacitance. Thus, measurements showing decrease in bulk capacitance over time enables us to monitor cell death.

Our objective is to demonstrate that the "detection by death" approach (which involves recording a loss of signal upon the death of microorganisms of interest) can indicate the presence of viable microorganisms of interest much faster than using traditional approaches based on detection of growth/metabolism. Hence, the most dramatic differences are likely to be observed in cases where the microorganism of interest is slow growing. One clinically important microorganism that takes a long time to be detected because of its long doubling time/slow metabolism is *Mycobacterium tuberculosis* (Mtb), which takes days (and sometimes weeks) to be detected using automated culture-based instruments like the BACTEC MGIT 960 (Becton Dickinson), MB/BacT ALERT system (bioMerieux), ESP Culture System II (Difco Laboratories) and Versa TREK Mycobacteria detection system (Versa TREK Diagnostics)[22].

Mtb is most commonly collected from the sputum of patients. Sputum samples obtained from tuberculosis-afflicted patients contain both mycobacteria as well as non-mycobacterial species like *Staphylococcus aureus*, *Pseudomonas aeruginosa,* etc.[23]. Thus, we created two types of artificial sputum: one containing *S. aureus, P. aeruginosa,* and mycobacteria, and the other containing the first two bacteria, but without any mycobacteria, to mimic the case of sputum of patients with and without TB. Also, we sought to create a control sample (no bacteria). The other possible case (mycobacteria present, but other bacteria absent), is not considered as relevant, since other commensal and pathogenic bacteria are invariably present in the sputum.

With most current culture-based systems like MGIT etc., one has to first "decontaminate" the sputum sample (eliminate all non-mycobacterial microorganisms present) before trying to assay for the presence of mycobacteria. There exist multiple standard protocols of digestion

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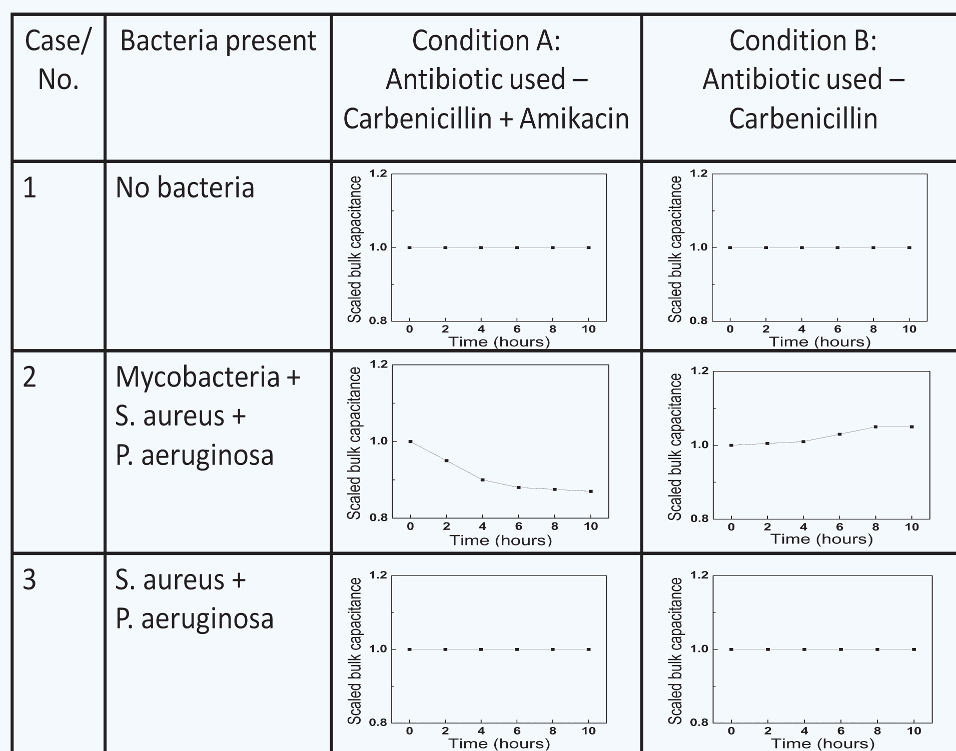

Figure 1 Qualitative illustration of the plots of bulk capacitance vs. time that we expect to obtain when exposed to two conditions post-decontamination (with carbenicillin and amikacin, and with carbenicillin alone) for the control (no-bacteria) and two possible cases likely to be encountered (mycobacteria present along with commensal bacteria and only commensal bacteria present).

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and decontamination for doing the same, and companies like Becton Dickinson, Hardy Diagnostics, etc. sell reagent kits designed to do so. It may also be noted that the decontamination process is subject to user errors that may result in a certain fraction of non-mycobacterial species surviving the process. So, to have a high degree of diagnostic sensitivity and specificity, next steps of the assay need to be designed accordingly.

Thus, in our next step, we treat the apparently decontaminated sputum samples by exposing them to two conditions as shown in **Fig. 1**. Under condition A, the samples are to be exposed to a cocktail of two antibiotics (amikacin and carbenicillin) while under condition B, the samples are exposed to carbenicillin only. Amikacin is known to have bactericidal effects towards *M. smegmatis*[24], *M. bovis BCG*[25] *and M. tuberculosis*[26]. Carbenicillin is known to be ineffective against mycobacteria but bactericidal against most other non-mycobacterial species[27]. As depicted, in **Fig. 1**, depending on the presence or absence of mycobacterial and non-mycobacterial species in the sample, the bulk capacitance of the suspension is expected to behave differently over time. If one observes death (decrease of bulk capacitance) in the presence of amikacin, it indicates the possible death of mycobacteria (and hence the presence of live versions in the original sample). (Not seeing a similar "death" signature in the presence of carbenicillin alone confirms that the death signature is not due to the presence of non-mycobacterial species.)

Also, since *Mycobacterium tuberculosis* is a Biosafety Level III (BSL-III) microorganism, we use *Mycobacterium smegmatis* and *Mycobacterium bovis BCG* as surrogate organisms to demonstrate proof-of-principle. *M. smegmatis* is a rapidly growing BSL-I organism with a doubling time of ~3 hours and has membrane characteristics very similar to *M. tuberculosis*[28,29] while *M. bovis BCG* is a slow growing BSL-II organism, whose doubling time of ~20 hours[15] is comparable to that of *M. tuberculosis* ~24 hours[28,29]. This is done to show that not only are we able to detect the presence of these organisms quickly using our approach but that the TTDs obtained on using our method are independent of the doubling time of the organisms.

METHODS

Overview

Figure 2 shows the summary of our experimental protocol. As shown in the **Fig. 2a**, we first create a sample of artificial sputum containing not only mycobacteria but gram-positive and gram-negative bacteria as well.

Initial loads of bacteria used are ~1×10^5 to 5×10^5 CFU/ml (maintaining a ratio of 1:1 between mycobacteria and other bacteria). A standard protocol for real-world samples of human sputum that involves the use of sodium hydroxide/N-acetyl-L-cysteine (NaOH/NALC)[30–32] is then used to digest and decontaminate the simulated sputum samples as shown in **Fig. 2b**. This treatment kills all bacteria other than mycobacteria in the sample. Post-decontamination and centrifugation, the sample is resuspended in fresh media and allowed to incubate at 37 °C for 2–3 hours as shown in **Fig. 2c**. To the media, antibiotic(s) are added, and thereafter, at regular intervals of time, small aliquots (~50 µl) are withdrawn, inserted into the thin channels of a microfluidic cassette and subjected to electric scans, as shown in **Fig. 2d**. Each scan involves applying a small AC voltage (500 mV) at multiple frequencies ranging from 1 kHz to 100 MHz across gold electrodes in contact with the suspension and recording the impedances at various frequencies. The data is processed to obtain an estimate of the bulk capacitance, a parameter that reflects the amount of charge stored by particles in the interior of the suspension and is thus correlated with the number of living microorganisms present. The manner in which the bulk capacitance changes over a few hours after the addition of the antibiotic(s) provides information on the presence of viable mycobacteria (microorganism of interest) in the original sample.

Details of the individual steps (including data collection, analysis, and interpretation) are provided below.

Bacterial cell cultures

For the *in vitro* study, we used either *Mycobacterium smegmatis* (ATCC® 700084™), or *Mycobacterium bovis BCG* (ATCC® 35734™). *Staphylococcus*

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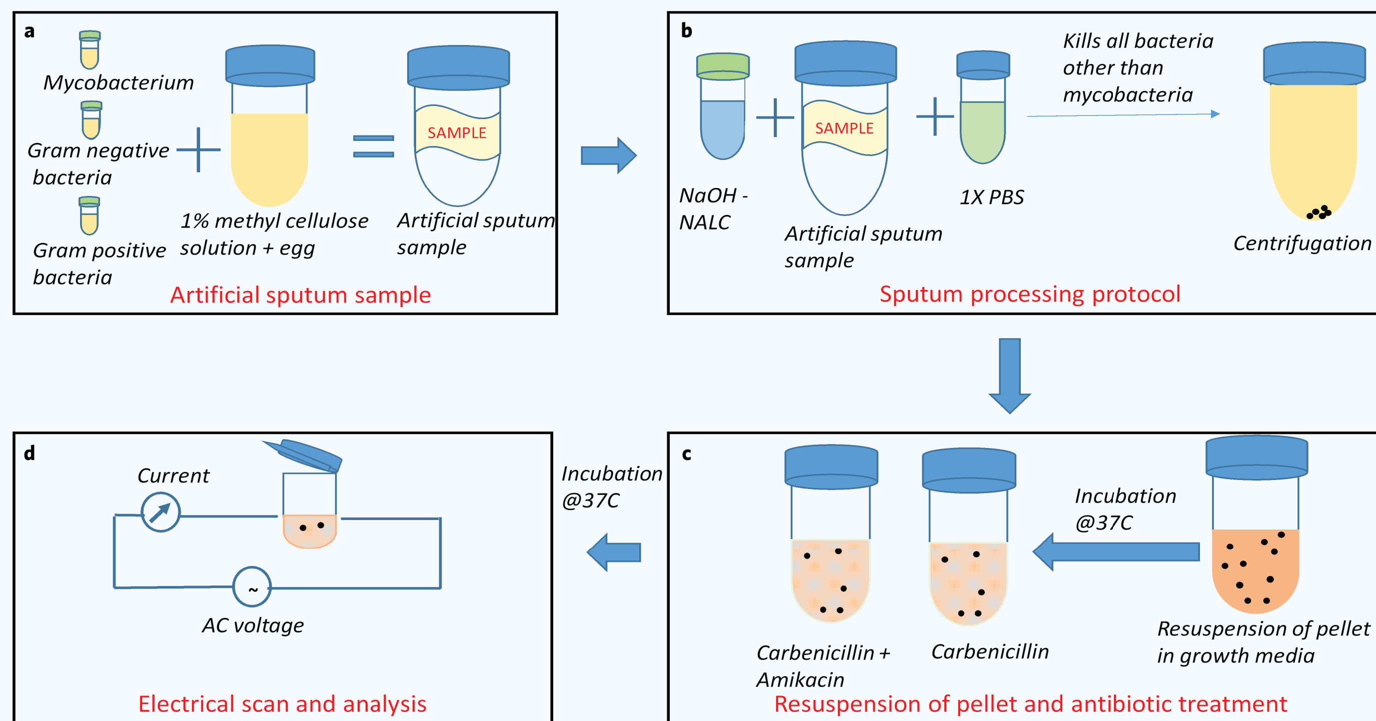

Figure 2 Experimental set-up to demonstrate the ability of the "detection by death" approach to detect the presence/absence of mycobacteria in a sputum sample. (**a**) Artificial sputum sample, containing both pathogen of interest (mycobacteria) and commensal bacteria (gram positive and gram negative), is prepared. (**b**) Standard protocol (treatment with NaOH-NALC) is used to liquefy the (artificial) sputum and decontaminate it (kill all non-mycobacterial microorganisms). The addition of PBS and centrifugation to obtain cells is also part of the protocol. (**c**) Collected cells are resuspended in two broths: one containing carbenicillin in 7H9 media, and the other containing both carbenicillin and ampicillin in 7H9 media. (**d**) At regular intervals of time (every hour), 50 µl aliquots are extracted and scanned electrically.

aureus (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853) are chosen as our model gram-positive and gram-negative organisms, respectively. *M. smegmatis* and *M. bovis BCG* are sub-cultured in Middlebrook 7H9 media supplemented with Middlebrook Albumin Dextrose Catalase (ADC) supplements at 37 °C. The optical density (OD) value for *M. smegmatis* is adjusted to $OD_{600} = 0.1$ and for *M. bovis BCG* is adjusted to $OD_{600} = 0.05$ using a spectrophotometer which corresponds to $\sim 1 \times 10^7$ CFU/ml and $(1\text{–}5) \times 10^6$ CFU/ml respectively[33,34]. All the other bacteria, other than mycobacteria are sub-cultured in Tryptic Soy Broth (TSB) at 37 °C to obtain log cultures. The OD value is adjusted to $OD_{570} = 1.5$ and $OD_{600} = 0.1$ which corresponds to 1×10^7 CFU/ml and 1×10^8 CFU/ml for *S. aureus* and *P. aeruginosa* respectively[35,36].

Digestion and decontamination

We use artificial sputum, which is prepared according to protocols available in the literature[37–39]. Briefly, 1 L of 1% (w/v) aqueous methylcellulose solution is prepared. After autoclaving the same, one emulsified egg is added. This artificial sputum is then used for our experiments. The sputum processing technique adopted is based on standard techniques that use N-acetyl-L-cysteine (NALC) to liquify and sodium hydroxide (NaOH) to decontaminate the sample[30,40,41]. Briefly, for each 100 ml of the solution, 50 ml of 0.5 N NaOH is combined with 50 ml of 0.1 M trisodium citrate solution and 0.5 g of powdered NALC; 10 ml of the NALC-NaOH solution is added to 10 ml of the sputum in a 50 ml tube and vortexed to mix. The solution is then allowed to stand at room temperature for 10 minutes. During this time the sputum is digested, decontaminated and liquefied. After this, phosphate buffered saline (1× PBS) solution is added to make up the volume of the solution to 50 ml. The addition of 1× PBS and the resulting dilution stops for all practical purposes the harmful action of the NaOH. Following this, the tubes are centrifuged at >3000 g for 15 minutes, the supernatant is decanted, and the pellet is re-suspended in 20 ml of fresh media.

Choice of antibiotics

Amikacin (32 µg/ml) is obtained from Fisher Scientific and carbenicillin disodium salt (25 µg/ml), is obtained from Research Products International Corporation. Initially, stock solutions (6400 µg/ml of amikacin and 5000 µg/ml of carbenicillin) are created by dissolving in DI water and desired concentrations (32 µg/ml for amikacin and 25 µg/ml of carbenicillin) in culture media obtained by appropriate dilution.

Microchannel Electrical Impedance Spectroscopy (m-EIS)

All m-EIS readings are conducted on artificial sputum samples that have been subject to digestion and decontamination using NALC-NaOH technique and then suspended in growth media laced with antibiotics.

The basic principles governing the use of m-EIS to detect microorganisms have been described in our prior work[7,9]. Briefly, we sense changes in bulk capacitance (C_b) by relying on geometric effects that enhance the effect of changes in C_b to the measured reactance (X) (the "imaginary" or "out-of-phase" component of the impedance). As shown in **Fig. 3b**, the use of long narrow microfluidic channel causes a larger fraction of the electrical flux lines to interact with the (few) microorganisms present. Another way to look at the effect is to study the equation embedded in **Fig. 3a**. Since for any given material, the resistance is inversely proportional to cross-sectional

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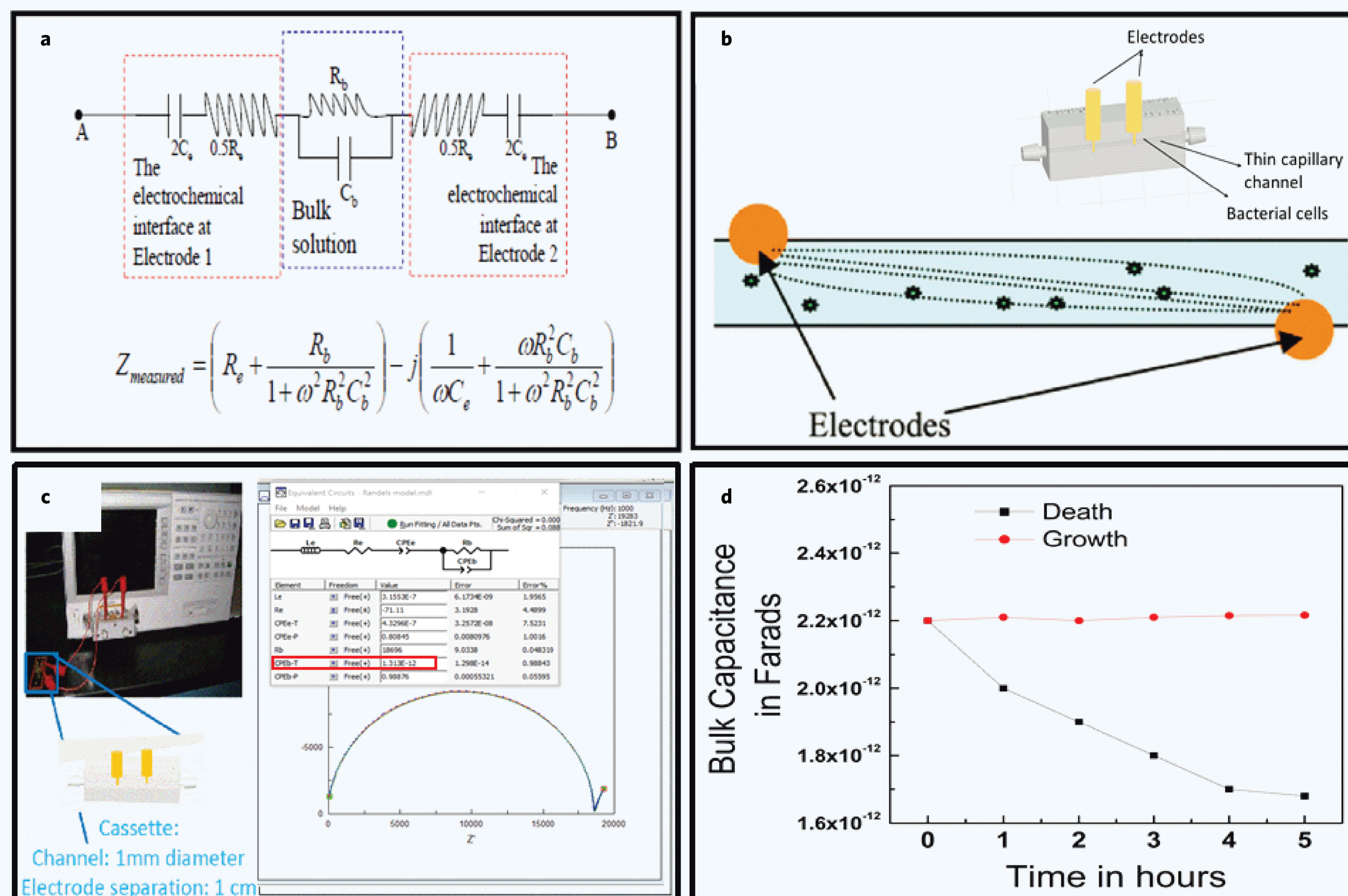

$$Z_{measured} = \left(R_e + \frac{R_b}{1+\omega^2 R_b^2 C_b^2}\right) - j\left(\frac{1}{\omega C_e} + \frac{\omega R_b^2 C_b}{1+\omega^2 R_b^2 C_b^2}\right)$$

Figure 3 (**a**) Electrical equivalent circuit model that represents our microfluidic cassette which is used for measuring the impedance of the bacteria. (**b**) Microfluidic cassettes with two gold electrodes inserted at a distance of 1 cm (inset) and schematic of electric lines of forces present between the two electrodes when an AC voltage is applied to the system[9]. (**c**) Agilent Impedance Analyzer is used to do the electrical scans at multiple frequencies and commercially available Z view® software is used to analyze the data to obtain the values for the various electrical parameters. (**d**) The bulk capacitance values obtained from data analysis is plotted against time. The decrease in the bulk capacitance values (black line) is due to cell death while the rise is due to bulk capacitance (red line) values is due to the cell growth.

area and directly proportional to length, the long narrow geometry results in an increase in bulk resistance (R_b). It can be seen that for the reactance (X), the C_b is always multiplied by R_b. Thus, any changes to the value of X due to a change in C_b will be "magnified" by the higher R_b. Since the R_bC_b is also multiplied by the frequency (ω), this effect is further enhanced at high frequencies. In addition, our electrical sensitivity is further enhanced by using AC signal with higher frequencies (ω) as high as 100 MHz. At these frequencies, the charge on the electrode reverses every ~10 nsec. A consequence of this is that there is not enough time for ions of opposite charge to completely cover the electrode, and thus the electric field is able to penetrate into the bulk to a greater degree and cause a greater degree of charge accumulation at the cell membranes.

Our experimental protocol requires us to periodically (every hour) perform an electrical "scan" of sample aliquots in a microfluidic cassette, wherein we measure electrical impedance at multiple (200) frequencies ranging from 1 kHz to 100 MHz. As shown in **Fig. 3b**, the cassette contains a 1mm diameter microchannel with two gold electrodes, 1 cm apart in the channel. An AC voltage of 500 mV is applied across the two gold electrodes, using an Agilent 4294A Impedance Analyzer. At each frequency (ω), both the in-phase and out-of-phase components of the electrical impedance, Z, (resistance (R) and reactance (X)) are measured. To take the EIS measurements (scans), all aliquots from a given culture (across the different points in time) are introduced into the same individual cassette. As the cassettes used are handmade their readings vary from each other slightly and hence the data (values of bulk capacitance obtained) is scaled with respect to the value at the initial point in time (on the same cassette) to account for the cassette-to-cassette variation.

The Z vs. ω data is fitted to an equivalent electrical circuit shown in **Fig. 3c** using a commercially available software package (Z-view™). The software provides an estimate for the various circuit parameters, including the "bulk capacitance", which happens to be 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 the bulk capacitance is represented as a constant-phase element (CPE) to account for the non-ideal nature of the capacitance at cell membranes. The magnitude of the CPE, thus, reflects the amount of charge stored at the membranes of living microorganisms in suspension. Any decrease in the number of microorganisms in suspension should hence, in theory, lead to smaller amounts of charge stored in the interior of suspensions,

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and hence lead to a lower bulk capacitance ($CPE_b - T$) over time as shown in **Fig. 3d**.

When trying to observe cell death in a suspension suspected of harboring living microorganisms, our problem reduces to asking the question of "Is the current value of the bulk capacitance *significantly* lesser than its value at the initial point in time?" To enable us to answer this question with a greater degree of confidence, for each sample, the capacitance of 4 replicates is measured at a specified time interval and statistically compared to baseline using Mann-Whitney U test. The earliest time-point at which a significant decrease is found is defined as the TTD for our "detection by death" method. Details of the statistical method are provided below.

Statistical analysis

Statistical analysis is performed in Microsoft Excel using Mann Whitney U-test. This non-parametric test compares if the population average between two groups is significantly different or not[42]. We chose to adopt the Mann-Whitney U-test over the more popular tools like t-test since we have only a few (4) data points (bulk capacitance readings) per time point. More importantly, the normality assumption of the reading that is required for a t-test is not appropriate for our data. To check if the average of the bulk capacitance obtained at a time interval is significantly different from the average bulk capacitance reading obtained in the first reading, the mean of the readings taken at the latter point in time is compared with the mean of the readings at the beginning of the culture (baseline values) and the U values corresponding to a p-value of 0.05 (level of significance of 5%; two tailed test) are calculated. Our null hypothesis is that the two bulk capacitance values are equal and the alternate hypothesis is that there is a significant difference between the bulk capacitance values. The Mann-Whitney U value obtained for our readings is compared to the critical U value[42]. If the Mann-Whitney U value obtained is equal to or less than the critical value (in this case, critical value = 0), the null hypothesis is rejected, which means that there is a significant difference between the bulk capacitance values at the two time points. The earliest point in time where the U values obtained are equal to, or lower than the critical U value is our time-to-detection (TTD) for a given sample.

RESULTS

As outlined in **Fig. 1**, three different cases are studied under two conditions. In Case 1, the sample has no bacteria. Hence, no changes in charge storage (bulk capacitance) occur at any point in time, and we expect to see a flat line as there should be no change in the bulk capacitance over time. In Case 3, where the sample contains gram-positive and gram-negative bacteria, but no mycobacteria, all organisms are killed during decontamination (pre-treatment) itself, and the addition of the antibiotics is not expected to cause any changes to the measured value of bulk capacitance. However, if there are mycobacteria in the sample (as in Case 2), they will survive the decontamination process and will continue to grow in the presence of carbenicillin (Case 2B). However, they will die in the presence of amikacin (Case 2A). This combination (dip in the presence of amikacin, but not in the presence of carbenicillin alone) will indicate the presence of mycobacteria. It may be noted that if the decontamination is done improperly, and some gram-positive and gram-negative bacteria survive, they will be killed under both conditions, and we can expect to see a dip in the bulk capacitance vs. time curve for both conditions.

Figure 4 represents the bulk capacitance values obtained when *M. smegmatis* is used while **Table 1** gives the corresponding U-values at the particular time intervals with respect to the baseline values. The initial loads of the bacteria used are (1 to 5) $\times 10^5$ CFU/ml. In the case of controls (Case 1A and 1B), we observe that there is no change in the bulk capacitance values over time and we get flat lines parallel to the x-axis. Also, the U-values calculated show that there is no significant difference between the bulk capacitances obtained at various time intervals. In Case 3A and 3B, the process of decontamination eliminates non-mycobacterial cells in the suspension and hence, in the absence of *M. smegmatis*, there is no significant change in the bulk capacitance values over time. For Case 2, condition A, where a cocktail of *M. smegmatis*, *P. aeruginosa,* and *S. aureus* is exposed to amikacin and carbenicillin after decontamination, we see that the impedance values show a decreasing trend over time, and the reading after 3 to 4 hours (depending on the experiment) is lower than the baseline value in a statistically significant manner. The decrease in the impedance values is due to the death of the remaining *M. smegmatis* in the presence of amikacin. Under condition B, a similarly decontaminated mixture of *M. smegmatis*, *P aeruginosa,* and *S. aureus* is not found to show any decrease over time. This is because, in the absence of amikacin, the mycobacteria present are not killed. It is possible that the mycobacteria actually grow during this time, but the growth rate is too slow for us to discern any increase in bulk capacitance in the observation timeframe.

Similar results are obtained, where the mycobacteria used is *M. bovis BCG*. **Figure 5** represents the bulk capacitance values obtained when *M. bovis BCG* is used while **Table 2** gives the corresponding U-values at the particular time intervals with respect to the baseline values. Here in Case 2A, decreasing bulk capacitance is observed after 1 hour itself, but no growth is seen in Case 2B during the duration of observation (3 hours). It may be noted that while we expect cells to be proliferating in Case 2B, the rate of increase in bulk capacitance is observed to be negligible. This is not surprising since the doubling times of the microorganisms is long (~20 hours for *M. bovis BCG* and ~ 3 hours for *M. smegmatis*), and in fact, underlines the advantage in speed of our method *vis-a-vis* growth-based detection approaches.

As mentioned earlier improper (incomplete) decontamination can lead to certain non-mycobacterial species surviving the decontamination step. This typically leads to false positives for culture (growth) based detection methods[5]. However, our approach provides a means to identify these false positives as well. If non-mycobacterial species are present in the sample after decontamination, death would be observed for both conditions A and B (unlike for condition A alone if decontamination is done correctly). To simulate a case of incomplete decontamination, samples of artificial sputum containing a cocktail of *S. aureus* and *P. aeruginosa* are exposed to NaOH-NALC for approximately 1 minute (as opposed to the 10 minutes previously used to achieve complete decontamination). Also, the NaOH concentration used is 0.25 N (as opposed to 0.5 N used to achieve complete decontamination). The sample thus obtained is exposed to antibiotics: both carbenicillin in combination with amikacin (condition A) and carbenicillin alone (condition B). As shown in **Fig. 6**, in such a situation, we observe decreases in bulk capacitance over time for *both* conditions, unlike when decontamination is complete, and mycobacteria are the only surviving live species (Case 2, condition A).

DISCUSSION

Thus here, we have (a) introduced the idea that live organisms can be detected by observing them die, (b) shown our ability to observe the death of organisms using m-EIS, and (c) outlined and implemented a scheme involving monitoring death (or lack thereof) of microorganisms in a sample upon exposure to two sets of antibiotics using which one may detect the presence of live Mycobacteria in sputum samples. The times to detection (TTDs) achieved using our method are 3 to 4 hours.

In theory, the concept of "detection by death" could be used with other methods of killing and/or other methods for monitoring the state (living or dead) of members of the cell-population of interest. For instance, one could kill the cells using gamma radiation, and monitor cell death using live/dead staining. Our choice of the killing agents (antibiotics amikacin and carbenicillin) is designed to bring about an identifiably distinct response when mycobacteria are present in a matrix known to harbor other kinds of gram-positive and gram-negative bacteria. Similarly,

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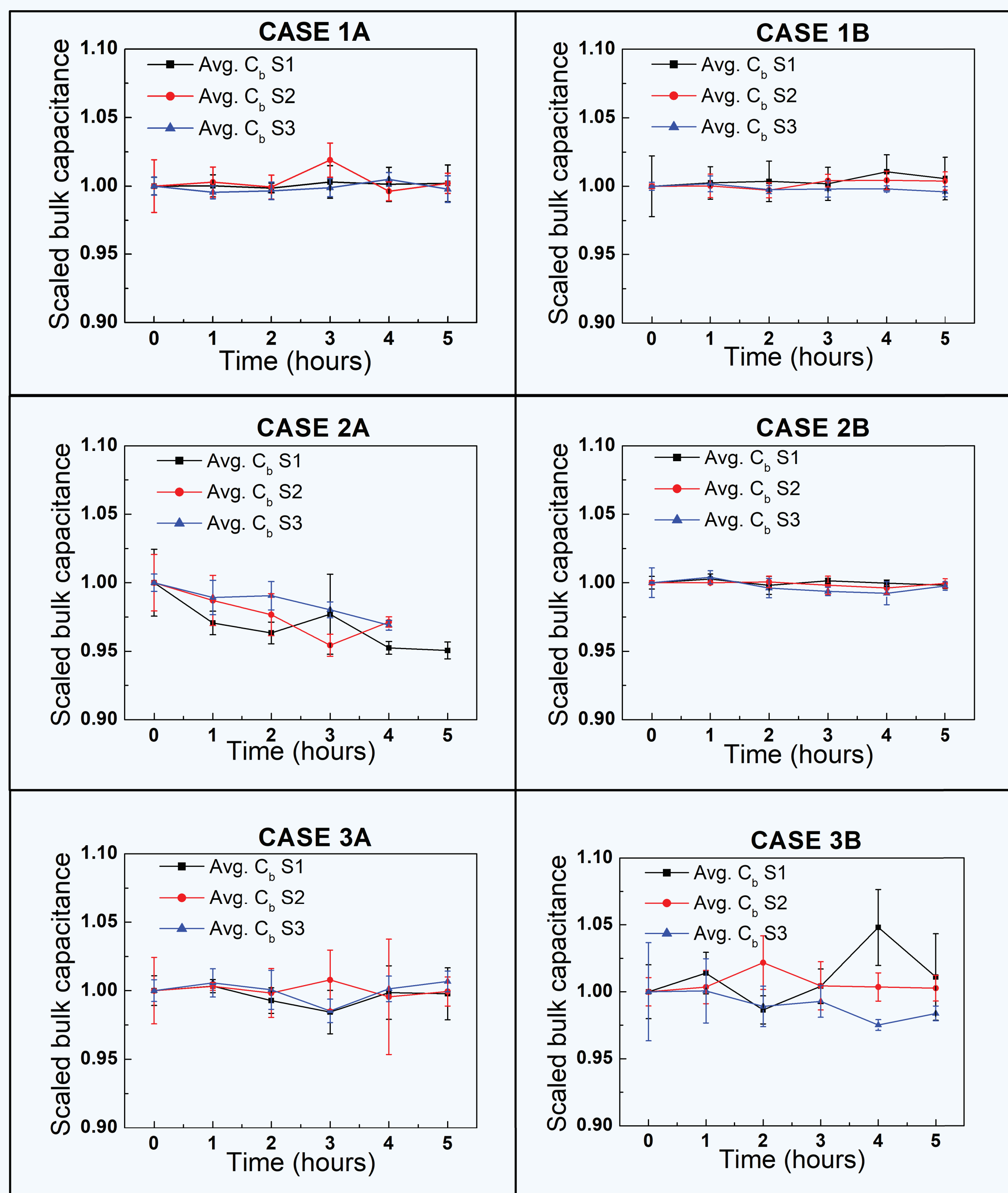

Figure 4 Three different cases of sputum sample (that have undergone decontamination) are exposed to two conditions namely, Condition A: cocktail of two antibiotics, amikacin and carbenicillin and Condition B: only carbenicillin. m-EIS scans are done to estimate the bulk capacitance values that enables us to detect the presence or absence of *M. smegmatis*. Average bulk capacitance values (C_b) vs. time is plotted.

the m-EIS method is chosen because (a) it can monitor cell proliferation/death in near-real time (scan times typically less than 2 minutes), (b) the AC signals that it uses (500 mV, 1 KHz to 100 MHz) does not harm the cells, which keep proliferating even upon exposure to the signals[7,12], and (c) requires very little action from the user (pipetting an aliquot of the sample into a micro-channel).

Even the little effort that is currently needed will no longer be required once the method is fully automated. A fully automated version of our

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Table 1 Statistical analysis of artificial sputum sample containing *M. smegmatis*, *S. aureus* and *P. aeruginosa*. Three different cases of sputum sample (that have undergone decontamination) are exposed to two conditions namely, Condition A: cocktail of two antibiotics, amikacin and carbenicillin and Condition B: only carbenicillin. Statistical analysis is done to estimate the Mann-Whitney *U* values *U*-values less than or equal to critical value (in this case, critical value = 0) indicates significant difference between the bulk capacitance values at that time interval with respect to the baseline reading and hence the presence of *M. smegmatis*. S = significant difference, NS = not significant difference.

CASE 1A								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	8	NS	1	7	NS	1	5	NS
2	6	NS	2	8	NS	2	5	NS
3	7	NS	3	4	NS	3	6	NS
4	5	NS	4	8	NS	4	5	NS
5	6	NS	5	8	NS	5	8	NS

CASE 1B								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	8	NS	1	8	NS	1	6	NS
2	7	NS	2	4	NS	2	5	NS
3	8	NS	3	4	NS	3	5	NS
4	6	NS	4	4	NS	4	4	NS
5	6	NS	5	4	NS	5	3	NS

CASE 2A								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	4	NS	1	6	NS	1	4	NS
2	2	NS	2	6	NS	2	6	NS
3	7	NS	3	0	S	3	0	S
4	0	S	4	0	S	4	0	S
5	0	S						

CASE 2B								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	5	NS	1	7.5	NS	1	5	NS
2	6	NS	2	5	NS	2	6	NS
3	7.5	NS	3	7	NS	3	5	NS
4	7	NS	4	5	NS	4	5	NS
5	6	NS	5	4	NS	5	7	NS

CASE 3A								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	6	NS	1	4	NS	1	5	NS
2	3	NS	2	8	NS	2	7	NS
3	4	NS	3	6	NS	3	2	NS
4	7	NS	4	7.5	NS	4	7	NS
5	7	NS	5	6	NS	5	4	NS

CASE 3B								
Sample 1			Sample 2			Sample 3		
Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline	Time (hours)	*U* value	Comparison with baseline
0			0			0		
1	5	NS	1	6	NS	1	6	NS
2	4	NS	2	3	NS	2	7	NS
3	7	NS	3	7	NS	3	8	NS
4	1	NS	4	7	NS	4	6	NS
5	6	NS	5	7	NS	5	8	NS

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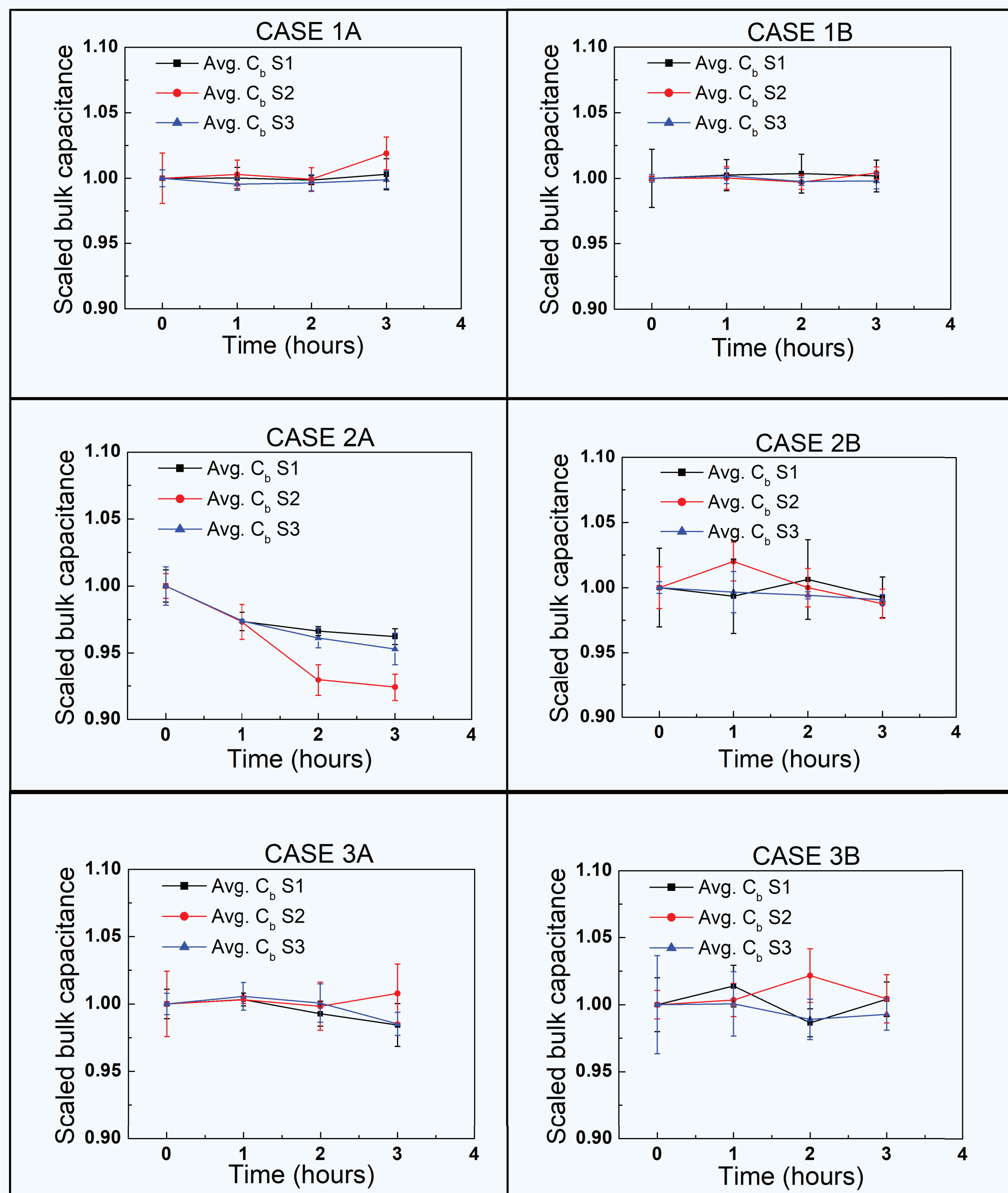

Figure 5 Three different cases of sputum sample (that have undergone decontamination) are exposed to two conditions namely, Condition A: cocktail of two antibiotics, amikacin and carbenicillin and Condition B: only carbenicillin m-EIS scans are done to estimate the bulk capacitance values that enables us to detect the presence or absence of *M. bovis* BCG. Average bulk capacitance (C_b) values vs. time is plotted.

method would consist of a fixed instrument (that would house the electric circuits used to conduct the m-EIS measurements), and disposables (cassettes/tubes with growth media and antibiotics). Such an automated system would, like the MGIT® and similar automated culture systems, be free from variability due to user judgement and inexpensive (not requiring expensive chemicals with strict storage requirements). In addition, our method can rule out a major source of false positives seen in traditional culture based methods (incomplete decontamination).

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Table 2 Statistical analysis of artificial sputum sample containing *M. bovis* BCG, *S. aureus* and *P. aeruginosa*. Three different cases of sputum sample (that have undergone decontamination) are exposed to two conditions namely, Condition A: cocktail of two antibiotics, amikacin and Carbenicillin and Condition B: only carbenicillin. Statistical analysis is done to estimate the Mann-Whitney U values U-values less than or equal to critical value (in this case, critical value = 0) indicates significant difference between the bulk capacitance values at that time interval with respect to the baseline reading and hence the presence of *M. bovis* BCG. S = significant difference, NS = not significant difference.

CASE 1A									CASE 1B								
Sample 1			Sample 2			Sample 3			Sample 1			Sample 2			Sample 3		
Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline
0			0			0			0			0			0		
1	8	NS	1	7	NS	1	5	NS	1	8	NS	1	8	NS	1	6	NS
2	6	NS	2	8	NS	2	5	NS	2	7	NS	2	4	NS	2	5	NS
3	7	NS	3	4	NS	3	6	NS	3	8	NS	3	4	NS	3	5	NS

CASE 2A									CASE 2B								
Sample 1			Sample 2			Sample 3			Sample 1			Sample 2			Sample 3		
Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline
0			0			0			0			0			0		
1	0	S	1	0	S	1	0	S	1	6	NS	1	2	NS	1	7	NS
2	0	S	2	0	S	2	0	S	2	7	NS	2	6	NS	2	1	NS
3	0	S	3	0	S	3	0	S	3	8	NS	3	4	NS	3	0.5	NS

CASE 3A									CASE 3B								
Sample 1			Sample 2			Sample 3			Sample 1			Sample 2			Sample 3		
Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline	Time (hours)	U value	Comparison with baseline
0			0			0			0			0			0		
1	6	NS	1	4	NS	1	5	NS	1	5	NS	1	6	NS	1	6	NS
2	3	NS	2	8	NS	2	7	NS	2	4	NS	2	3	NS	2	7	NS
3	4	NS	3	6	NS	3	2	NS	3	7	NS	3	7	NS	3	8	NS

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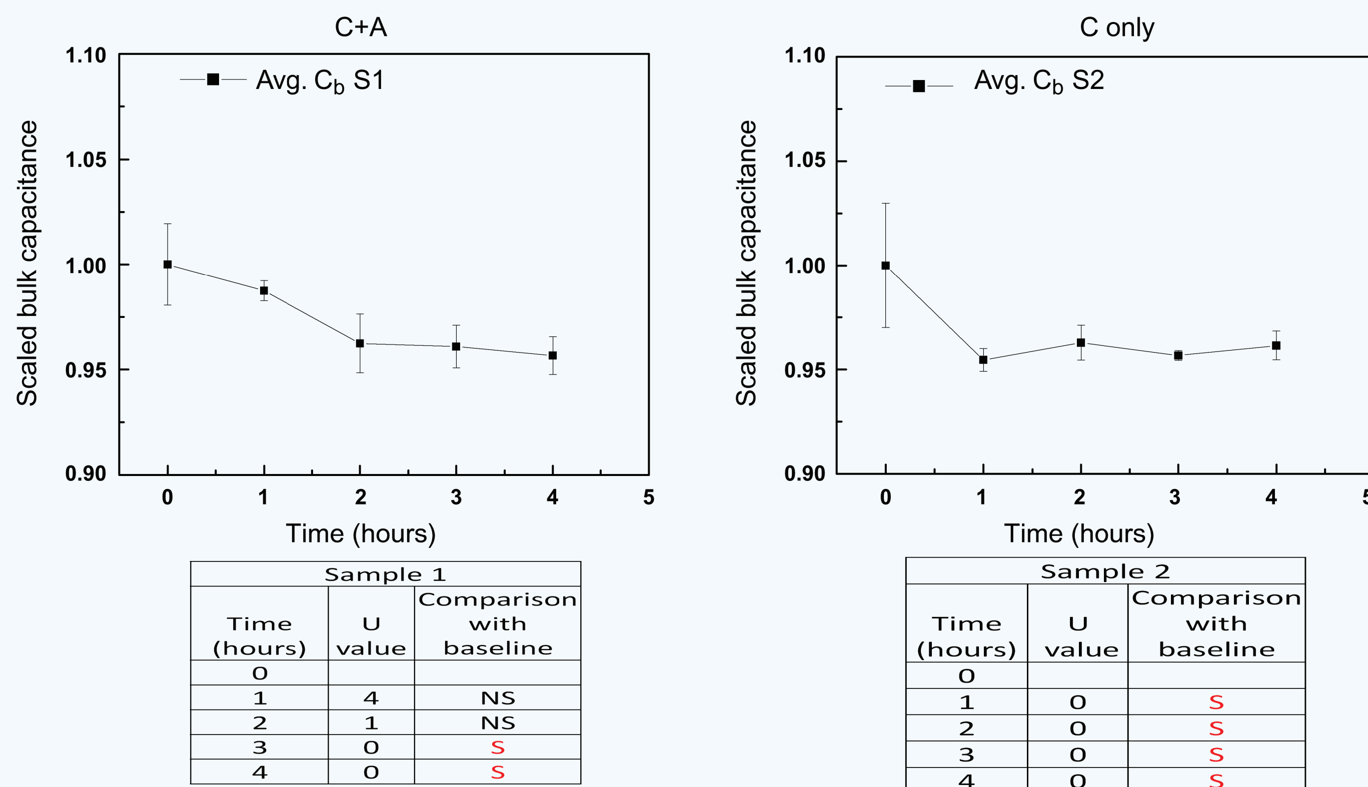

Sample 1		
Time (hours)	U value	Comparison with baseline
0		
1	4	NS
2	1	NS
3	0	S
4	0	S

Sample 2		
Time (hours)	U value	Comparison with baseline
0		
1	0	S
2	0	S
3	0	S
4	0	S

Figure 6 Partial decontamination of simulated sputum samples containing non-mycobacterial cultures are exposed to two conditions namely, Condition A: cocktail of two antibiotics, amikacin and carbenicillin and Condition B: only carbenicillin. Average bulk capacitance estimated from the m-EIS scans is plotted against time.

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In contrast, because they are not related to the doubling times/metabolic rate of organisms, our TTDs compare extremely favorably with those of culture-based detection methods. In fact, regarding TTD, our method is comparable to those obtained by the latest "molecular" (DNA-based) methods like the Xpert® MTB/RIF. The XpertMTB/RIF instrument (with four modules that can run 16–20 tests per 8-hour shift) has a cost of ~US$34,000, and each single use cartridge has a cost of ~US$40. However, a consortium of charitable organizations that include the Foundation for Innovative New Diagnostics (FIND), the Gates Foundation, USAID and UN agencies, provide a 50% subsidy on the instrument and a 75% subsidy on the disposables to "approved" public health agencies in low-resource countries, making the Xpert™ instrument available for US$17,000 and disposables available for ~US$10 a test[43].

Thus an automated system implementing our method is likely to combine the benefits of culture based systems (low cost and ruggedness) with those of the new "molecular" systems like the Xpert®, viz. the rapid times of detection of a few hours. If successful in doing so, it is well positioned to compete against not only the current gold-standards, but also many of the other emerging technologies that are being developed for the diagnosis/detection of active tuberculosis (especially pulmonary TB) and/or assay for (multi) drug resistance. These emerging technologies have been reviewed comprehensively by others[44,45], and include a variety of approaches such as Nucleic-Acid Amplification Tests, immunological tests, rapid culture systems, bacteriophage-based assays etc.

In view of the general difficulty in comparing various diagnostic approaches, participants at a 2013 TB Modelling and Analysis Consortium (TB MAC) proposed a different approach to evaluating new diagnostics for TB[46]: viz. by proposing Target Product Profiles (TPPs). TPPs state the clinical purpose of a test (e.g. triage, confirmatory testing, treatment monitoring etc.), implementation level in the healthcare system (rural clinics, large hospital etc.) and target end users. Based on expert feedback, nine TPPs were identified as relevant, and assigned a priority rank. The top ranked TPP was for a "Rapid, sputum-based, molecular test for microscopy centers (with the option of add-on drug susceptibility testing cartridge)". While, our test is not molecular per se, its turnaround time is comparable to other molecular tests, and it can be potentially implemented in current microscopy centers (typically, banks of simple microscopes located in a rural clinic or district hospital operated by lab technicians who visually inspect sputum smears). Also, monitoring behavior against TB drugs other than amikacin will yield information on drug resistance. It is also likely to be well suited to other TPPs as well, such as (a) triage test for those seeking care (priority # 3, joint), (b) an HIV/ART clinic based test to rule out active TB (priority # 3, joint), and (c) systemic screening test for active case finding (priority # 5).

Thus, we believe our technology (once automated) has the potential to meet multiple niches in the TB diagnostics world. Of course, we would have to build an automated system to implement our approach, as well as demonstrate that our method works on *M. tuberculosis* present in actual human sputum (instead of *M. bovis BCG* and *M. smegmatis* in artificial sputum). We plan to do both in the near future.

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