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Rapid antibiotic susceptibility testing by resazurin using thin film platinum as a bio-electrode

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ABSTRACT

Traditional antibiotic susceptibility testing methods take several days to confirm and start disease treatment. The lack of new antibiotics or drugs warrants a need for optimization of current diagnostic tools for immediate antibiotic susceptibility testing. We present a rapid screening method to evaluate the response of bacteria to antibiotics based upon the electrochemical measurement of live bacterial cell metabolic activity using an electroactive redox dye, resazurin. A thin film of Pt deposited over a glass substrate using the direct current sputtering technique was used as a working bio-electrode for electrochemical readouts. X-ray diffraction and scanning electron microscopy was carried out to characterize the Pt thin film. We tested the efficacy of the method using two different strains, *Klebsiella pneumoniae* (ATCC-700603) and *Escherichia coli* (ATCC-25922), against ampicillin, kanamycin and tetracycline. The dye, on incubation with viable bacteria, undergoes reduction and lowers the corresponding peak current value. However, in the presence of an effective antibacterial agent, reduction did not occur due to bacterial cell death and absence of a reducing environment. The electrochemical changes in peak current values were monitored using the differential pulse voltammetry technique and interpretation of the results obtained was based upon changes in peak current values. Our results depict a new methodology where a concentration of 10^4 cells/mL cells can be detected in less than 4 h. The results were also compared with the conventional disc diffusion method for susceptibility testing which has a bacterial incubation time of 18–24 h. The method can potentially be used for monitoring the susceptibility of bacterial strains towards existing antibacterial agents in an easy, rapid, reliable and inexpensive manner without any pre-cultivation of bacteria.

1. Introduction

Identification of an infectious agent and the best antibiotic for its treatment within a typical eight-hour working day is difficult. Traditional methods of antibiotic susceptibility testing require several days to start treatment. Therefore, physicians use broad-spectrum antibiotics to begin the treatment and control the disease from further spread. However, the approach is disadvantaged by the occurrence of antibiotic resistance and a resurgence in deaths. The frequency at which antibiotic resistance is increasing, is an alarming situation and a matter of concern globally. Various common pathogens that have already acquired resistance include *Pseudomonas aeruginosa*, *Escherichia coli, Shigella, Salmonella typhi*, *Acinetobacter* spp. These infectious agents are the most frequent causes of serious diseases including the very common ones such as urinary, genitourinary, respiratory and gastointestinal infections (Khaledi et al., 2016; Collignon, 2009). Among the number of causes contributing to antibiotic resistance the most common ones include improper usage of antibiotics, delay in diagnosis of bacterial infections and lack of public healthcare awareness. (Kakkar et al., 2017). Susceptibility testing is especially important for bacterial species that are more prone to either develop or acquire resistance (Reller et al., 2009). It is also very important in the context of bioterrorism or during

Abbreviations: AST, Antibiotic susceptibility testing; CV, Cyclic Voltammetry; DPV, Differential Pulse Voltammetry; EIS, Electrochemical Impedance Spectroscopy; Pt, Platinum; DW, Distilled water; MHA, Mueller Hinton agar; MHB, Mueller Hinton broth; DC, Direct current; XRD, X-ray diffraction; SEM, Scanning electron microscopy; CLSI, Clinical and Laboratory Standard Institute; CDC, Centre for Disease Control; POC, Point of care

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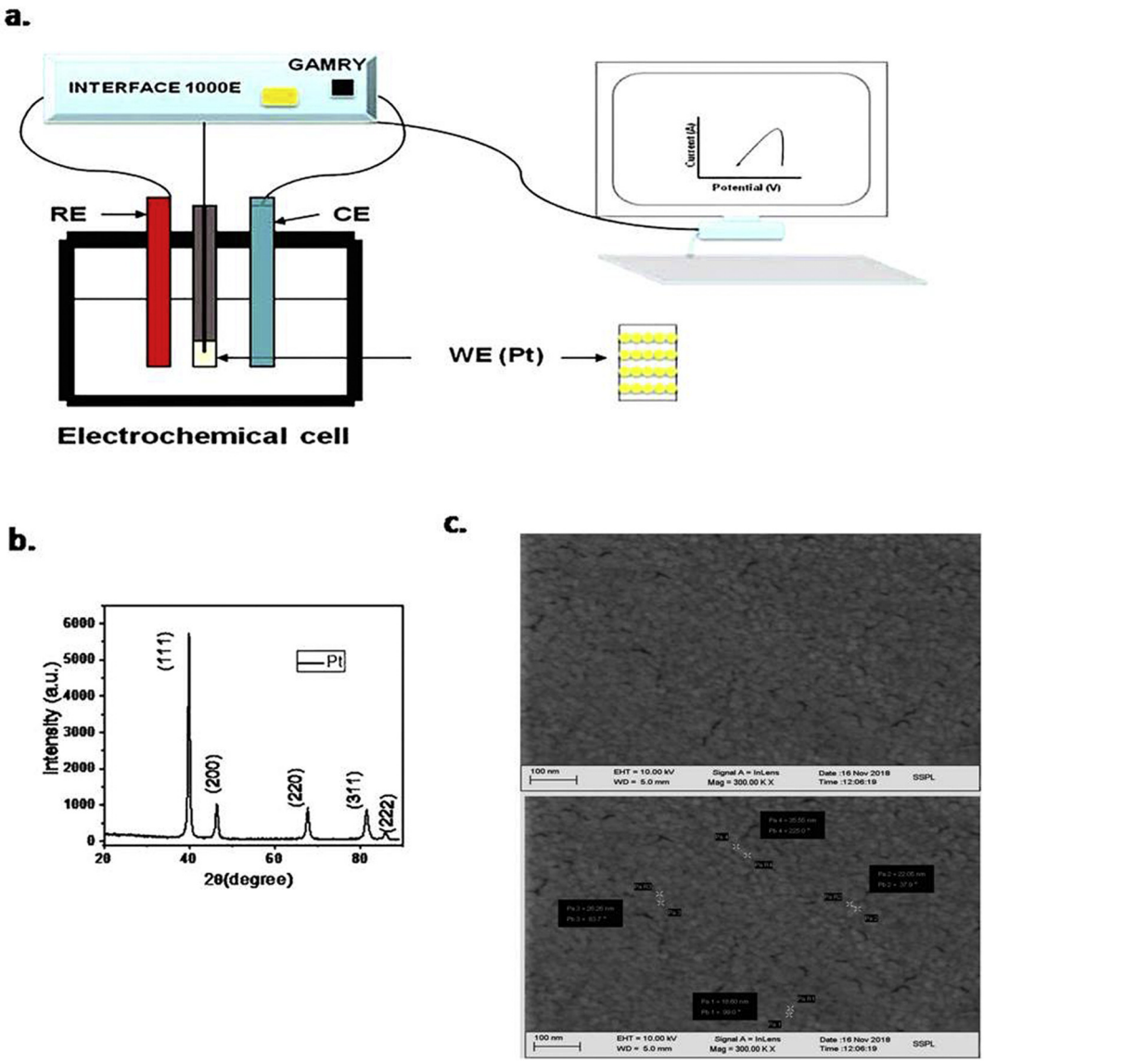

Fig. 1. (a) A schematic representation of the electrochemical setup showing working electrode (WE), counter electrode (CE) and reference electrode (RE), (b) XRD spectra of the Pt thin film, (c) SEM images of the Pt thin film.

biological warfare as spread of antibiotic resistance could be the reason of bioterrorism (Dorsch, 2007). Thus, rapid diagnostic platforms offer relevance to enhance the sensitivity, specificity and cost of the assay.

The gold standard method for susceptibility testing includes disc diffusion and broth microdilution (Bauer et al., 1966; Villanova et al., 2004). These methods are inexpensive but have limitations in terms of AST time and skills. Several other methods are also used in clinical microbiology based upon colorimetric, spectroscopic, microscopic, cytologic and amplification based analysis with certain limitations. Other methods to address the issue include biosensing and microfluidics based approaches for rapid identification and AST. Different electrochemical techniques are in use to perform such bioassays (Webster et al., 2015). Some of them include CV, DPV and EIS (Nakamura et al., 1991; Besant et al., 2015; Puttaswamy et al., 2018). Others include impedence, capacitance and conductance of cells in growth medium that can also be used for antibiotics susceptibility testing (Huang et al., 1998) and pathogen detection (Gómez et al., 2002). Abeyrathne *et al* have used impedence based approaches for rapid and label free detection of AST in *S. aureus* using antibody functionalized interdigitated transducers within a time duration of 2 h (Abeyrathne et al., 2016). Such methods form the basis of various biosensing strategies for pathogen detection using microelectrodes fabricated on chips. These biosensors are important for biosecurity issues (Stephen and Evangelyn, 2005).

In the present study, we have demonstrated the innovative role of DPV, an electrochemical technique for rapid and efficient AST using electrodeposited Pt thin film as a working electrode. The role of Pt as a working electrode in electrochemical studies has been explored earlier (Aschauer et al., 1995; Pogacean et al., 2014). Performance of Pt thin film used during the experiment shows long-term stability at room temperature, reproducibility and offers ease of preparation. Resazurin present in the medium acts as an external mediator for transfer of electrons to the electrode. Thus, the current work demonstrates a simple, rapid and effective platform for antibiotic susceptibility testing.

2. Experimental section

2.1. Media preparation

MHA (R70191-5G; Sigma-Aldrich) and MHB (R70192-500G; Sigma-Aldrich) were used for antibiotic susceptibility testing as per the CLSI protocol. A stock solution of 10 mM resazurin (R7017-5G; Sigma-Aldrich) was prepared in sterile, autoclaved DW. The solution was

dissolved and vortexed to ensure the formation of a homogenous solution. A working concentration of 0.25 mM resazurin was used in bacterial growth medium.

2.2. Bacterial strains

The bacterial strains used in the study were *E.coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) obtained from All India Institute of Medical Sciences (AIIMS), New Delhi, India.

2.3. Antibiotic preparation

The stock solutions of ampicillin (171254-5GM; Calbiochem) and kanamycin (420311-5GM; Calbiochem) were prepared in DW while tetracycline (CMS2199-5G; Himedia) was prepared in 70% ethanol. The stock solution was stored at −80 °C and thawed at 4 °C for use in the experiment. Antibiotics were further diluted in DW and were added in broth to achieve the desired concentration of 10 μg/mL.

2.4. Electrochemical setup

The electrochemical measurement was conducted on Gamry Potentiostat/Galvanostat/ZRA Interface1000 using a conventional three-electrode setup comprised of Ag/AgCl as the reference electrode, Pt foil as the counter electrode and DC sputtered Pt thin film as the working electrode. All the measurements were performed in the culture medium without using any other buffer solution. A schematic representation of the electrochemical setup is shown in Fig. 1a.

2.5. Preparation and characterization of working electrode

The Pt thin film of thickness 100 nm was deposited over corning glass substrate of size 2 cm × 2 cm using the DC sputtering technique. The substrate was thoroughly cleaned prior to deposition *via* subsequent ultrasonication for 10 min each in trichloroethylene, acetone and isopropyl alcohol. The sputtering chamber was pumped down to a vacuum level of 10^{-6} mBar before the sputtering process and then deposition was carried out in 100% argon ambience maintained at the chamber pressure of 0.02 mBar. A DC power of 20 W was used to sputter out a high purity (99.999%) Pt metal target. To improve the adhesion of the Pt thin film, a titanium (Ti) buffer layer of 10 nm thickness was *in-situ* deposited before Pt on the substrate. Thus, as prepared Pt/Ti/glass system was used as the working electrode for electrochemical measurements. Characterization of the prepared Pt thin film was carried out

by XRD (XRD PANalytical XPert PRO MRD) and SEM (ZEISS, SUPRA™55, FESEM).

2.6. Bacterial culture and propagation

The bacterial colonies of different strains were transferred under aseptic conditions into a 10 mL MHB containing capped conical flask and incubated overnight at 37 °C. After 18–24 h of incubation, cells were centrifuged at 6000 rpm for 5 min, supernatant was discarded and cell pellet was resuspended in PBS followed by centrifugation. This removed debris and a clean bacterial suspension was obtained followed by suspending cells in MHB. The absorbance of the bacterial suspension prepared was recorded by UV–Visible spectrophotometer at 600 nm (OD_{600}). The cells were adjusted in the range of 0.15 to 0.2 OD_{600} which was considered to have cells at a concentration of 10^8 cells/mL. This suspension was further diluted to obtain a concentration of 10^7 cells/mL for testing antibiotics by disc diffusion method.

2.7. Antibacterial susceptibility test of antibiotics

The standard AST performed was agar disc diffusion method as per the guidelines from CLSI (Villanova et al., 2004). For agar disc diffusion, MHA plates were inoculated with log-phase bacterial cell suspension of *K. pneunomiae* and *E. coli* at the concentrations described in Section 2.6. Sterile filter paper discs were placed on the agar plates after which they were soaked with 10 μg of different antibiotic solutions (kanamycin, ampicillin, tetracycline). The plates were further incubated for 18–24 h at 37 °C in an incubator. The antibacterial susceptibility was interpreted based upon appearance of a circular zone of inhibition. The experiment was repeated thrice in order to confirm the susceptibility profile of antibiotics against bacterial strains used.

2.8. Characterization of redox resazurin dye

Electrochemical response of dye was accomplished using DPV. The electrochemical nature and peak currents of the dye were initially observed in 0.01 M phosphate buffer saline. Initially a wide potential range of −0.8 to −0.0 V was taken to locate the reduction/oxidation peaks. We then shifted the potential window in the range of −0.6 to −0.1 V. The dye generates one major peak at a potential range of −0.2 to −0.3 V and one minor peak between −0.3 and − 0.4 V. In this range the dye was further characterized and peak currents were observed at different concentration of 0.125, 0.25, 0.5 and 0.9 mM. A glass electrochemical cell was used to carryout the measurements.

2.9. DPV based antibacterial screening of bacterial strains against different antibiotics

Further the bacterial cell suspensions of *K. pneumoniae* and *E. coli* for AST were prepared as described in Section 2.6. The final concentration of the cells in tube was adjusted to 10^4–10^5 cells/mL aseptically. The antibiotics to be tested were further added at a final concentration of 10 μg/mL excluding the controls. The tubes were further incubated at 37 °C for 1 h to ensure the action of antibiotics on bacterial growth after which resazurin at a concentration of 0.25 mM was added and DPV scans were recorded in the standardized potential window of −0.6 to −0.1 V. DPV was performed as a function of the concentration of resazurin, as metabolic activity of viable cells provides a reducing environment for continuous metabolization of the dye present in the medium containing cells. The DPV spectra was observed in the standard potential range for MHB alone, MHB inoculated with bacterial cells and MHB containing different antibiotics using the same bio-electrode without any external mediator or dye but no peak current was observed. A decrease in the current was noted upon incubation of bacterial cells with antibiotics indicating loss of metabolic activity due to presence of antibiotic resistance. However, a constant current indicated bactericidal activity of antibiotics.

2.10. Reusability of bio-electrode

The reusability of the fabricated bio-electrode was investigated by observing DPV scans of resazurin dye mutiple times in different culture medium. A single bio-electrode was used throughout the experiments.

3. Results

3.1. Structural and morphological characterization of working electrode

The XRD pattern of the deposited Pt thin film (Fig. 1b) showed characteristic peaks at 39.8°, 46.5°, and 68.2° which may be attributed to (111), (200), and (220) planes of Pt (Aaltonen et al., 2004). It was observed that the deposited Pt film is highly (111) oriented. The SEM image (Fig. 1c) reflected that the surface of the Pt thin film deposited over the glass electrode had a dense and uniform morphology.

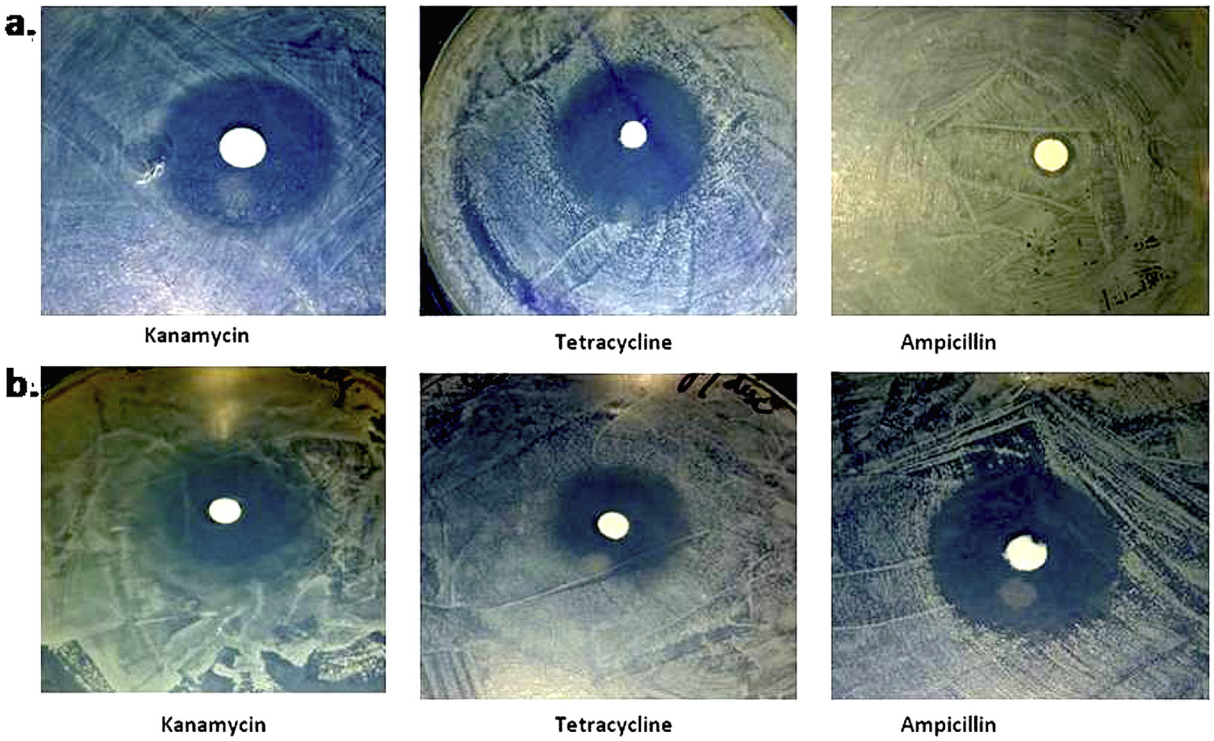

Fig. 2. Antibiotic susceptibility profile of (a) *K. pneumoniae* and (b) *E. coli* to ampicillin, kanamycin and tetracycline at a concentration of 10 μg/mL using disc diffusion method.

3.2. Antibacterial activity of antibiotics

The susceptibility results of *K. pneumoniae* and *E.coli* using the conventional method indicated that *K. pneumoniae* was susceptible to tetracycline and kanamycin but resistant to ampicillin, whereas *E.coli* was susceptible to all 3 antibiotics (Fig. 2).

3.3. Antibiotic susceptibility screening using DPV

An increase in the peak oxidation current was observed from 5.6 to 19.6 μA on increasing the concentration of resazurin dye from 0.125 to 0.9 mM (Fig. 3). The concentration of 0.25 mM resazurin was selected for further experiments as significant increase in current was observed in comparison to the control (MHB alone). The changes in peak currents at different cell concentrations in a time dependent manner indicated decrease in current proportional to the bacterial cell concentration. This change was observed in 2 h with 10^6 cells/mL and 3 h with 10^4 cells/mL. However, no changes were observed in 10^2 cells/mL upto 5 h, thus an optimum concentration of 10^4 cells/mL was used for the AST experiments (Fig. 4). DPV scans for *K. pneumoniae* (Fig. 5A) and *E. coli* (Fig. 5B) showed a time dependent decrease in current at different time intervals in the absence of antibiotics in the growth medium. However, in the presence of ampicillin, a betalactam antibiotic, a decrease in current was observed. The results indicated resistance of the strain to ampicillin. However, when the strain was treated with kanamycin and tetracycline no change in current was observed (Fig. 5A, a–d). The voltammograms of *E. coli* when exposed to different antibiotics showed no changes in current indicating the killing action of antibiotics or susceptibility of the strain to antibiotics (Fig. 5B, a–d). The peak current values were plotted at different time intervals showing the time dependent changes in current values (Fig. 6). These results were compared with the disc diffusion method, thus, validating the applicability of the electrochemical method for rapid antibacterial screening. A direct comparison of the susceptibility profile of the antibiotics against

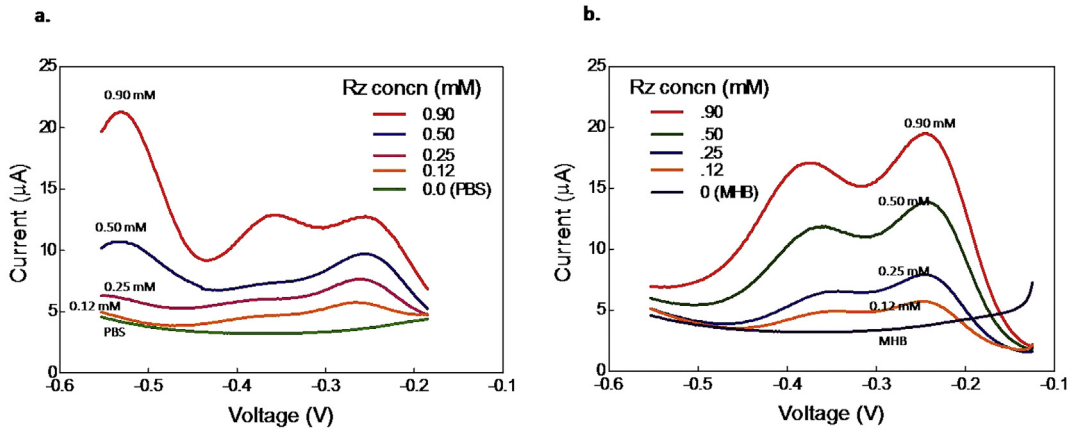

Fig. 3. DPV curves obtained at different concentrations of resazurin in (a) PBS solution (b) MHB. (Figures are representative of 2 independent experiments).

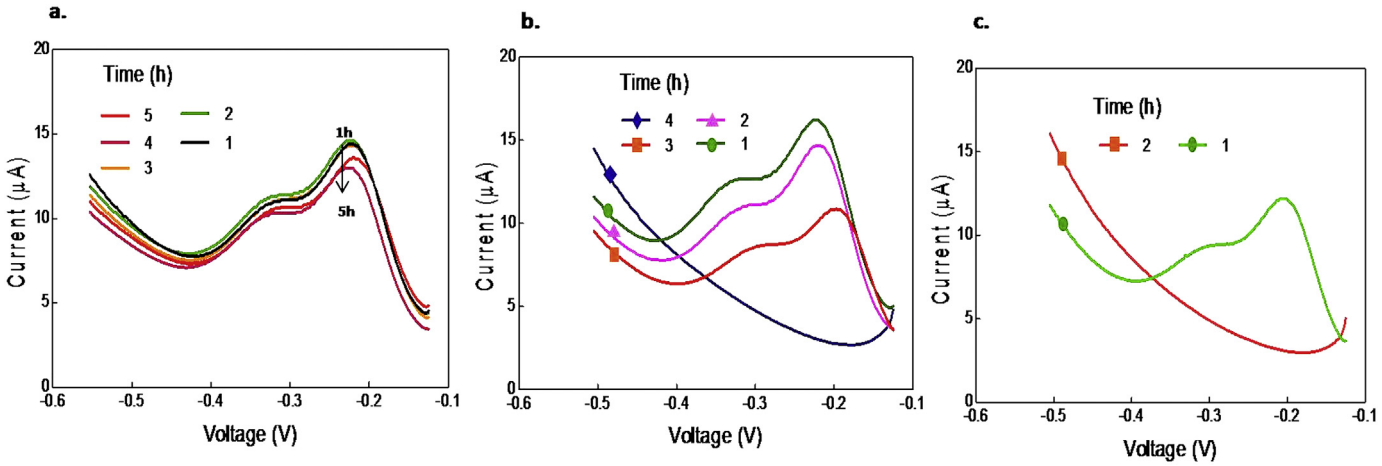

Fig. 4. Peak current observed as a function of time at different concentrations of (a) 10^2, (b) 10^4 and (c) 10^6 cells/mL. (Figures are representative of 2 independent experiments).

tested bacterial strains using conventional method and resazurin based electrochemical method is shown in Table 1. It was concluded that *K. pneumoniae* was susceptible to kanamycin and tetracycline while *E. coli* was susceptible to ampicillin, kanamycin and tetracycline.

4. Discussion

During the past few years, several reports have been published using different methodologies for rapid antibiotic susceptibility testing (Liu et al., 2017; Dong and Zhao, 2015; Pulido et al., 2013; Chen et al., 2017; Mezger et al., 2015). Although, the methods were successful in shortening the incubation period compared to the traditional disc diffusion method, high cost limited their routine use. Here, we present a rapid, reliable and inexpensive method for detection of antibiotic susceptibility by resazurin dye using DPV. Samples with 10^4 cells/mL can be detected in less than 4 h, and thus, no pre-cultivation is needed for biological samples.

E. coli and *K. pneumoniae* are the most common pathogens causing various nosocomial infections according to CDC. The most common ones include genitourinary infections, surgical wound infections, gastrointestinal as well as respiratory infections. These infections are often caused by breaching of infection control practices and procedures, unclean and non-sterile environmental surfaces or sick employees (Khan et al., 2015). In future, the pace of increasing resistance will definitely cause irreversible consequences for human health care. So there is a need for rapid diagnostic methods. The change in peak current due to bacterial cell viability after antibiotic exposure highlights the applicability of the electrochemical approach for low cost, rapid antibiotic susceptibility testing.

As in an electrochemical process, the electrode plays a major role in realizing input and output current values and the properties of the electrode play a substantial role in the success of electrochemical analysis (Li and Miao, 2013). Therefore, the use of highly sensitive, stable, low cost and easily fabricated electrodes demonstrates the advantages of various biosensing based approaches. Voltammetry is a very promising electrochemical technique to measure current as a function of applied potential. The technique offers measurements with high sensitivity, applicability, possibility of multicomponent analysis and analyte determination at various oxidation states, low primary investment and running cost. (Navratil et al., 2009). Thus, the method described here employs the use of DPV as an efficient electroanalytical technique using DC sputtered thin film Pt as a bio-electrode. This offers applicability in antibiotic susceptibility determination in 2 different bacterial strains in an easy rapid and cost effective manner using a redox dye. Redox dyes find their application in various cell based bioassays, immunological assays, energy transfer-based assays and microarray based assays (Nishi et al., 2015). One such dye is resazurin that is a blue colored, electroactive, redox dye (Rampersad, 2012). Microscopy has shown that the dye can penetrate cells, where it undergoes a reduction reaction as a result of the action of several different oxidoreductase enzymes inside the viable, metabolically active cells (Khazalpour and Nematollahi, 2014; Çakir and Arslan, 2010; Sarker et al., 2007). The ability of cells to reduce the dye depends upon incubation period and cell concentration. Our data also supports a time dependent decrease of peak current with respect to cell concentration in the presence of resazurin. It was also clearly observed that the decrease in peak current attributed due to reduction of the dye in the presence of a reducing environment of viable bacterial cells. However, in the case of antibiotic induced cell killing, resazurin remains unreduced and the characteristic peak current remains the same. Tetracycline, ampicillin and kanamycin used in the study are some of the commonly used antibiotics listed by WHO as essential medicine. In electrochemical experiments, a biorecognition element is primarily required to transfer or shuttle the electrons to the electrode surface in an efficient manner (Kaur et al., 2017). However, in our study the use of the dye within the medium eliminates the need for immobilization of the biorecognition molecule like strain-specific antibodies onto the electrode surface which was previously a key step in biosensor development that increases the cost of the method. The current method thus provides an easy, inexpensive and rapid way of AST that is faster than conventional methods which require 18 to 24 h after bacterial isolation.

This preliminary study requires an electrochemical setup, however in the near future the rapid response method could be easily miniaturized into a handheld portable biosensing device for POC applications in rapid susceptibility testing. Such electrochemical detection methods offer ease of miniaturization (Escamilla-Gómez et al., 2009) with applicability in field conditions and remote areas. This would assist clinicians to administer specific narrow spectrum antibiotics in bacterial infections instead of broad spectrum antibiotic usage that decreases the severity of infection but increases the risk for various health related side effects.

5. Conclusion

Our study demonstrates detection of bacterial susceptibility to antibiotics using an electrochemical technique. The results illustrate that the technique used will provide us a future outlook for rapid AST. The use of Pt coated bio-electrode serves as a promising candidate for application as a biosensor in analyzing the metabolic activity of viable bacterial cells, thus monitoring the response of different antibiotics on

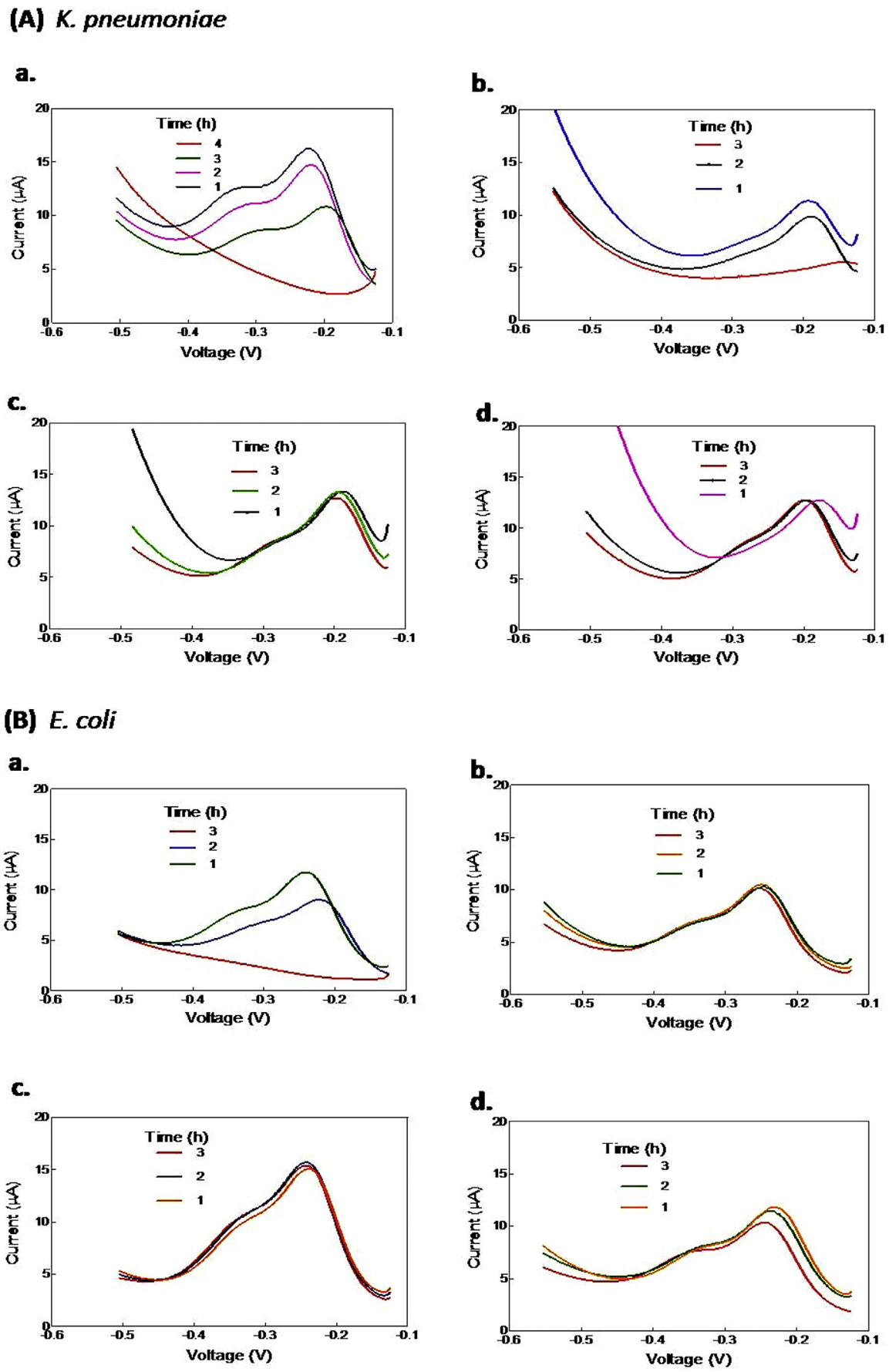

Fig. 5. DPV curves obtained at different time intervals in the presence of resazurin for (A) *K. pneumoniae* and (B) *E coli*. The antibiotic treatment (10 μg/mL) groups are represented as (a) Untreated control (no antibiotic), (b) ampicillin, (c) kanamycin, and (d) tetracycline. (Figures are representative of 3 independent

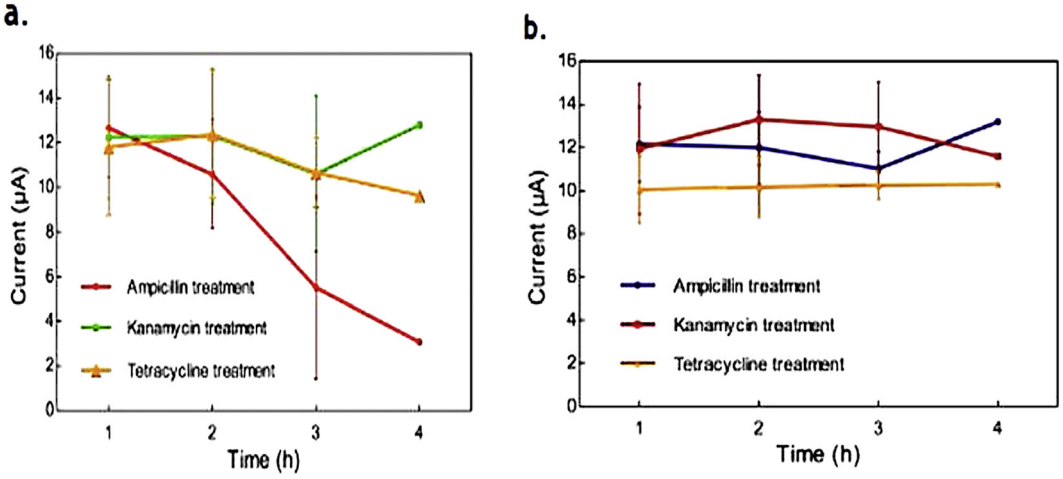

Fig. 6. Plots of the DPV peak current at different time points in MHB containing different antibiotics as indicated in figure when inoculated with (a) *K. pneumoniae* and b) *E. coli.*

Table 1
Comparison of susceptibility profile of different antibiotics obtained from conventional and DPV based electrochemical method.

	E. coli		*K. pneumoniae*	
	Conventional	DPV based AST	Conventional	DPV based AST
Ampicillin	Susceptible	Susceptible	Resistant	Resistant
Tetracycline	Susceptible	Susceptible	Susceptible	Susceptible
Kanamycin	Susceptible	Susceptible	Susceptible	Susceptible

bacterial cell viability. Consequently, the deposited Pt thin film is capable of detecting current changes in presence of redox molecule resazurin in less than 4 h. The approach could provide rapid antibiotic susceptibility profile that can help in starting antibiotic therapy early to reduce the progression of bacterial infections. The method can be further converted into a biochip for POC testing.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

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