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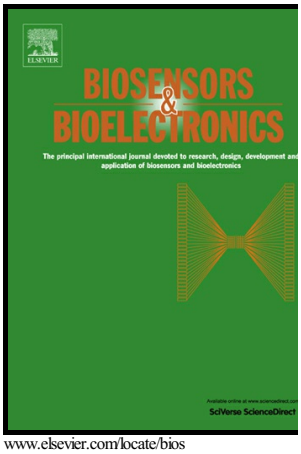

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A biosensing strategy for the rapid detection and classification of antibiotic resistance

Qun Chen [a,b,1], Anneli Andersson [b,2], Michael Mecklenburg [c] and Bin Xie [b*]

a. *Institute of Endemic Diseases, School of Public Health, Xi'an Jiaotong University Health Science Center, Key Laboratory of Trace Elements and Endemic Diseases, National Health and Family Planning Commission, 710061, Xi'an, China*

b. *Department of Pure and Applied Biochemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden*

c. *BT Biomedical Technology AB, SE-22738 Lund, Sweden*

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*Corresponding author. Department of Pure and Applied Biochemistry, Lund University, Getingevägen 60, POB 124, SE-221 00 Lund, Sweden, Tel: +46 (0)46 2228261, Fax: +46 (0)46 2224611, E-mails: bin.xie@tbiokem.lth.se (B. Xie)

Present addresses:

1. Institute of Endemic Diseases, School of Public Health, Xi'an Jiaotong University Health Science Center, Key Laboratory of Trace Elements and Endemic Diseases, National Health and Family Planning Commission, 710061, Xi'an, China

2.. Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich, CH-8057 Zurich , Switzerland

Abstract

Antibiotic resistance (AR) poses an ever growing threat to global public health. Methods are urgently needed that simplify and accelerate the clinical detection and classification of AR. Here we describe a function-based antibiotic resistance assay (FARA) biosensing strategy. The scheme comprises three key components: i) FARA directly measures the thermal signal generated from the catalytic break-down of antibiotics by AR enzymes, ii) a sample specific AR profile is created by analysing a panel of antibiotics which enhances informational content and iii) meta-analysis of the AR profile database to correlate profiles with diagnosis, treatments and outcomes. In order to test the ability of the scheme to identify and classify AR, two well-studied antibiotic resistance enzymes, penicillinase and metallo-beta-lactamase (MBL), were profiled using a panel of 5 antibiotics: penicillin G, penicillin V, ampicillin, oxacillin and imipenem. The results show that the profiles of the two enzymes could easily detect AR and differentially classified these enzymes. More importantly, both enzymes showed a significant and distinct secondary catalytic profile, which dramatically increases informational content. FARA profiles can be generated and analyzed in 1h. FARA is a fast, simple, cost effective alternative for detecting and classifying AR. FARA will speed up AR detection and classification will allow more accurate individualized treatment. This will reduce the spread of resistance and personalized treatments will improve patient outcomes. Other potential applications of FARA technology are discussed, including the possibility of developing an *in vitro* blood model for studying AR.

Keywords: β-lactamase; antibiotics; antibiotic resistance, thermal biosensor, profiling

1. Introduction

Antibiotic resistance (AR) poses a serious threat to global public health. The production of antibiotic degrading enzymes is the most common form of antibiotic resistance (AR). Resistance has even developed against the latest broad spectrum carbapenem antibiotics. Moreover, the extended-spectrum β-lactamases are not inhibited by therapeutic β-lactamase inactivators (Nordmann *et al.*, 2012). As a result, AR is a major cause of medical treatment failure (Bush, 2010). As one example, infections by carbapenemase producing *Enterobacteriaceae* are associated with mortality rates in the 20% to 40% range (Souli *et al.*, 2011). The recent isolation of mega AR plasmids containing multiple resistance genes, combined with a general increase in horizontal transmission and AR persistence, indicates that the battle to control the spread of AR is being lost (Johnson and Woodford, 2013; Andersson and Hughes, 2011). With few new antibiotics against Gram-negative bacteria in advanced clinical trials, there is an urgent need to find ways to slow the spread of AR (Freire-Moran *et al.*, 2011).

The development of faster, more accurate assays for detecting and classifying AR would significantly reduce misdiagnosis and allow the use of narrow spectrum antibiotics, thereby reducing the use of broad spectrum antibiotics. The classic disk assay remains the most widely used method for detecting AR due to its simplicity, low cost and minimal technical requirements (Bauer, *et al.*, 1966). Since the early 1980s, a variety of techniques for detecting AR have been developed, but the disk method remains the gold standard. New physical characterization methods with high information content have been developed based on microarrays, PCR and MS technologies (Sibley *et al.*, 2012; Hrabak *et al.*, 2013; Card *et al.*, 2013). And while these new techniques can rapidly and accurately identify AR, they require significant technical expertise and have considerable start-up costs which hinder their implementation. A number of rapid tests have also been developed (Dortet *et al.*, 2013; Longo

et al., 2013). These provide fast AR results for a number of specific diseases, but are impractical and uneconomical for large scale screening. With over 1600 β-lactamases sequenced to date and new variants constantly emerging, the development of a simple, fast method for identifying AR remains extremely challenging (Sibley *et al.*, 2012). New analytical strategies for detecting AR are urgently needed.

The ability of the disk method to ask specific functional questions makes up for the low information content the assay provides. This sharply contrasts with high information content complex assays such as nucleic acid and proteomic based AR detection methods provide. However, despite the low information content, the simple disk test remains the most widely used method for diagnosing AR due to its ability to provide specific clinically relevant information. The main drawback of the disk method is speed, with test results taking 24-48h. This often times forces the clinician to make a therapeutic decision before test results are available, which increases the risk of inappropriate therapies and over-use of antibiotics. The disk method detects the enzymatic break-down of antibiotics by measuring the extent to which microbial growth is inhibited. If a faster, more direct method for detecting the break-down of antibiotics could be substituted for microbial growth based detection, while retaining the simplicity of the assay, this would revolutionize AR testing.

One approach would be to develop a function-based antibiotic resistance assay (FARA) that profiles the substrate specificity of antibiotic degrading enzymes using biosensor technology. Enzyme biosensors are uniquely capable of detecting biomarkers with catalytic activity. As a result, a wide range of enzyme based biosensors have been developed (Nakamura and Karube, 2003; Narsaiah *et al.*, 2012; Ispas *et al.*, 2012). Our group has focused on developing biosensors that employ thermal transduction to directly detect the thermal signal generated during enzyme catalysis. The instrument, the enzyme thermistor (ET), has been used to develop a wide range of assays (Xie and Danielsson, 2007). Recently

we have shown that the device is capable of quantifying antibiotics in whole blood without any sample pretreatment (Chen *et al.*, 2013). In this scheme, the thermal signal generated when β-lactamase degrades antibiotics is used to quantify the amount of antibiotic present in blood. Based on this work, we have developed a FARA biosensing scheme capable of detecting and classifying AR by profiling the substrate specificity of β-lactamase enzyme(s). Here we present data showing that FARA profiling is able to detect and classify two AR enzymes, penicillinase and metallo-beta-lactamase (MBL).

2. Materials and methods

2.1 Chemicals and materials

Imipenem was bought from Fresenius Kabi (Bad Homburg, Germany). Penicillinase (EC 3.5.2.6) from Bacillus cereus, metallo-beta-lactamase (EC 3.5.2.6) from Enterobacter cloacae, penicillin G potassium salt, penicillin V potassium salt and Ampicillin were purchased from Sigma-Aldrich Co (St. Louis, USA). Propylamino-derivatized controlled-pore glass beads (CPG) were obtained from Steinachglas (particle diameter 125-140 μm, pore diameter 50nm, Steinach, Germany). All other reagents were purchased from Sigma-Aldrich Co (St. Louis, USA).

2.2 Buffers and sample preparation

A 100 mM sodium phosphate buffer, adjusted to pH 7.0 (hereafter phosphate buffer) and a modified PBS buffer which contained NaCl 137 mM, KCl 27 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaF 52 mM and EDTA 3.4 mM adjusted to pH 7.4 (hereafter PBS) were prepared. All water used for preparing the buffers and samples was from a Milli-Q system (Millipore, Bedford, MA, USA).

A stock solution of each antibiotic was prepared in phosphate buffer. The samples used to prepare the calibration curves were serially diluted in phosphate buffer. Venous blood from healthy volunteers was collected in EDTA-coated tubes and stored at 4°C.

2.3 Instrumental set-up

The Enzyme Thermistor (ET) biosensor used in this study was obtained from Omik Bioscience AB in Sweden. The ET is a flow-injected calorimeter. This model employs a dual column configuration (Fig. 1). The measurement column contained the CPG immobilized enzyme, while the other column acted as a reference column and contained CPG immobilized enzyme that has been inactivated (Chen *et al.*, 2013). The working temperature for the ET was set to 25°C. A flow rate of 0.5ml/min and a sample volume of 100µL were used unless otherwise stated. At least 4 repeated measurements were performed for each sample.

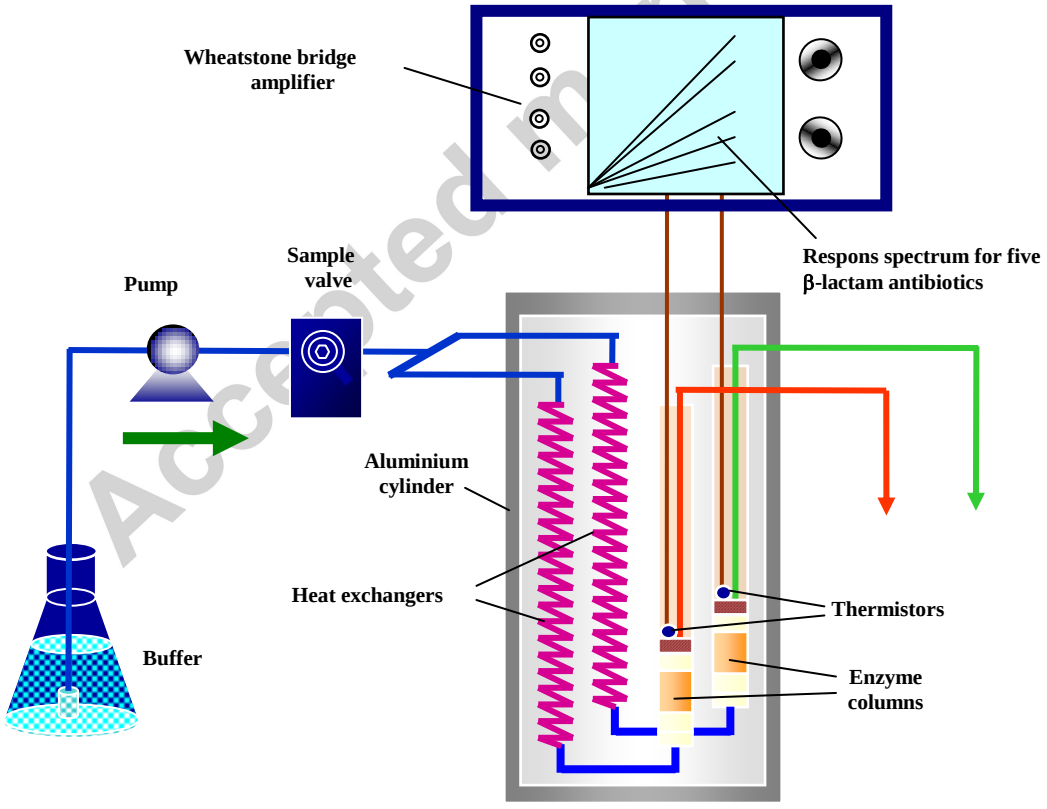

Fig. 1. Schematic of the Enzyme Thermistor biosensor.

2.4 Enzyme column preparation

Immobilized enzyme was prepared as follows: 700 mg CPG was combined with 10 ml of 2.5% glutaraldehyde in 100 mM sodium phosphate buffer were combined and allowed to incubate at room temperature under reduced pressure for 30 min, after which the incubation was continued for an additional 30 min at ambient pressure. The activated CPG beads were extensively washed, after which penicillinase (50 units) and MBLs (5 units) were added to the activated beads in separate glass tubes containing phosphate buffer. The coupling was allowed to proceed at 4°C under agitation overnight. Enzyme-bound CPG were washed and remaining sites were blocked by the addition of 2 ml coupling buffer containing 0.2M ethanolamine. After 2 h incubation at ambient temperature, the CPG was washed and then packed into a column.

3. Results and Discussion

3.1 Assay strategy

The aim of these studies was to lay the groundwork for a function-based AR assay, FARA. Conceptually the strategy combines the accuracy of function-based assays with the simplicity of the disk method. The primary drawback of the microbial-based growth detection with a faster method that detects the thermal signal generated during the catalytic breakdown of antibiotics. Direct detection of the thermal signal generated during antibiotic degradation provides a fast, simple method that eliminates the need for labelling and complex post reaction product analysis. Thermal based detection is only possible for enzymes that fulfil a number of criteria, i) exothermic reactions, ii) good stability, iii) limited cofactor requirements and iv) high turnover rates. β-lactamases are ideally suited for this assay strategy. And while the informational content of the assay is low, the test results provide clinicians with the specific information required to determine the most effective course of antibiotic treatment.

Moreover, the thermal substrate profile scheme allows the detection and identification of emerging as yet unidentified AR enzymes.

3.2 Development of Penicillinase and MBL of assays

In order to determine if FARA was capable of detecting and classifying AR enzymes, two β-lactamases were immobilized and profiled using the FARA protocol. Penicillinase and MBL were chosen for these initial studies because they are well studied and have very distinct antibiotic specificity profiles (Citri, 1971; Pfeifer, et al. 2010). The effect of various flow rates on assay sensitivity were tested. Flow rates of 0.5, 0.7 and 1.0 ml/min were analyzed. The optimal flow rate was determined to be 0.5ml/min (data not shown). In order to determine the effect of pH, studies were performed at three additional pH values: 6.5, 7.5 and 8.0. The results showed that the optimal response for penicillinase determinations was pH 6.5, which corresponds to published pH optimum between pH 6.0-6.5 for the enzyme. For MBL, optimal activity was obtained at pH 7.0. The linear regression values and slopes for both enzymes were similar over the entire pH range (data not shown). A pH of 7.0 was chosen for these studies.

Using these parameters, calibration curves for penicillinase and MBL were prepared employing penicillin G as the substrate (Fig. 2). The linear regression values for the penicillinase and MBL curves were 0.99985 and 0.99959, respectively. The slope values for the penicillinase and MBL curves were 0.002847 and 0.001727, respectively. Calibration curves for penicillinase and MBL were generated using blood spiked with penicillin G (Fig. 3). The linear regression values for the penicillinase and MBL blood calibration curves were 0.9983 and 0.9997, respectively. The slope values for the penicillinase and MBL curves were 0.002689 and 0.002769, respectively. The linear ranges and sensitivities for both enzymes are similar to that seen in buffer samples. However, the MBL activity was reduced, while the

penicillinase activity was largely unaffected. The recovery for penicillinase was 87.8% and 91.23% for MBL, respectively.

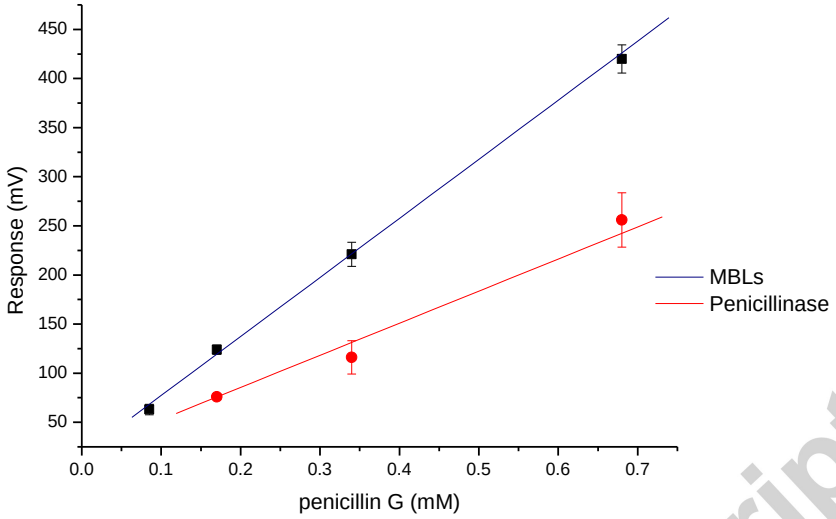

Fig. 2. Penicillin G calibration curves for penicillinase and MBL in phosphate buffer at pH 7.0.

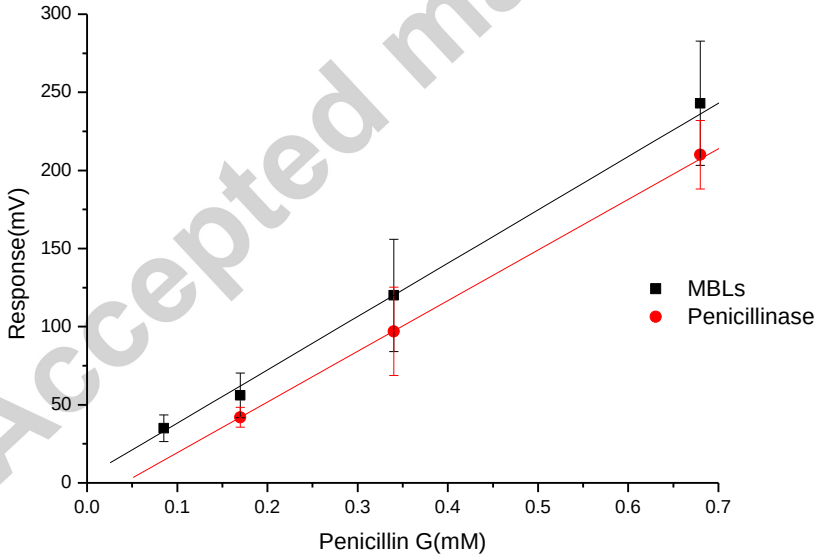

Fig. 3. Calibration curves for penicillinase and MBL using blood samples spiked with penicillin G.

The calibration curves for the immobilized enzymes are linearly and highly reproducible both in buffer and whole blood. Our results showed that the pH optima for penicillinase and MBL were 6.5 and 7.0, respectively. This is in accordance with previously published values. And while the activities of the enzymes vary somewhat with pH, the difference was not appreciable over the pH range tested. The pH of blood lies in this range, so the FARA blood analysis is not expected to be noticeably affected. Repeatability studies indicate that the analytical system is stable. The calibration curve slopes for penicillinase were largely the same in buffer and blood, while the slopes for MBL differed significantly. Reason for the shift seen with MBL is not known, but is highly reproducible and does not affect the sensitivity of the assay. Blood values of critically ill patients can vary widely and it will be important to perform extensive control studies using mock blood samples from healthy and ill patients to ensure test accuracy.

3.3 Repeatability Studies

Repeatability studies were performed using MBL at various penicillin G concentrations in buffer and spiked blood samples. The RSD values for MBL using buffer samples containing 0.17, 0.34, 0.68 and 1.36 mM penicillin G were 8.71, 6.71, 3.81 and 3.36%, respectively (Fig. 4). Repeatability studies for penicillinase and MBL using whole blood samples spiked with penicillin G at a single penicillin G concentration were also carried out. The RSD value for penicillinase using a concentration of 1.36 mM penicillin G was 6.68. The MBL RSD value using a concentration of 0.34 mM penicillin G was 10.89 (data not shown).

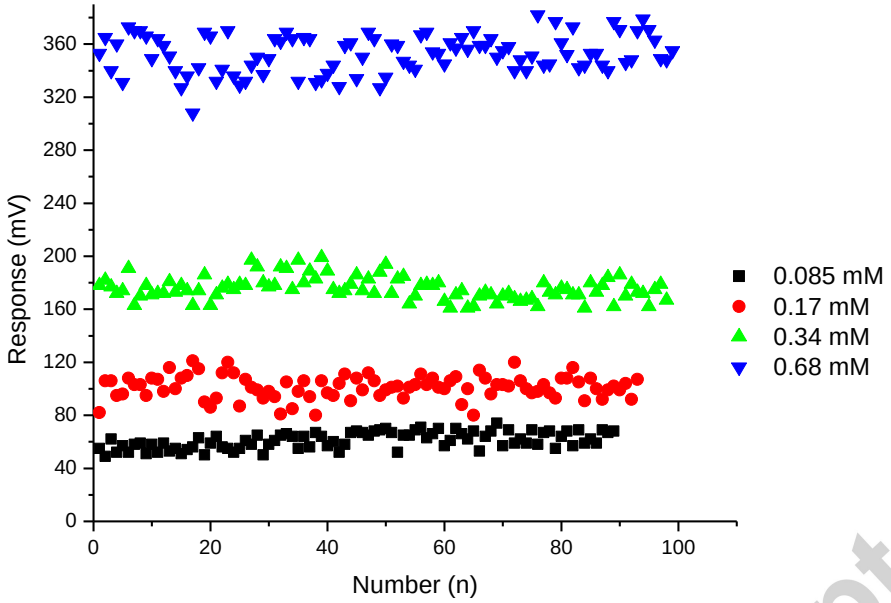

Fig. 4. Repeatability of MBL assay using penicillin G as substrate in buffer.

The results indicate that the assay is highly reproducible with excellent long term stability. As expected the RSD values for spiked blood samples was significantly higher than those obtained for the buffer samples, but nevertheless within acceptable limits. It should be noted that the mock blood samples were not pretreated in anyway, which normally results in unacceptable repeatability values. At the conclusion of these studies, the column had been used to analyse approximately 600 buffer samples and 300 unpretreated blood samples over a period of 3 months. In terms of performance, no observable deterioration was detected. Consequently, it is difficult to provide an estimate of column life, but clearly the column is capable of being used for numerous tests over an extended time period.

3.4 Enzyme substrate specificity profiling

Five antibiotics were chosen for FARA profiling of penicillinase and MBL: penicillin G, penicillin V, ampicillin, oxacillin and imipenem. Penicillin G and V were chosen as

positive controls since most, if not all, β-lactamases have significant activity against these antibiotics. Ampicillin and oxacillin were chosen because they are representative second generation antibiotics. Imipenem, a third generation antibiotic, was chosen as a representative of the broad spectrum carbapenem antibiotics to which AR has only recently developed.

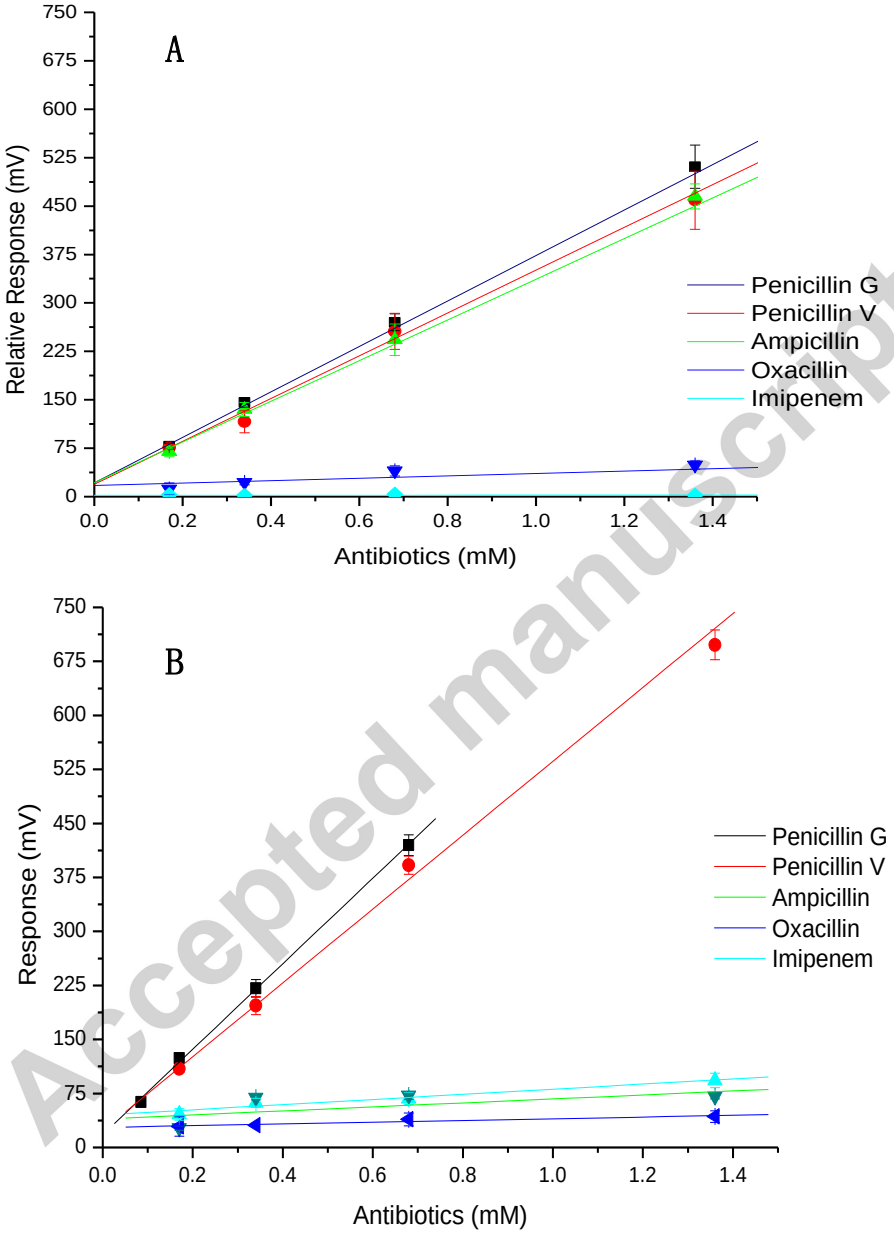

Fig. 5. Calibration curves for penicillinase (A) and MBL (B) using the antibiotic panel in buffer.

Calibration curves for penicillinase (Fig. 5A) and MBL (Fig. 5B) were prepared against the following panel of antibiotics: penicillin G, penicillin V, ampicillin, oxacillin and

imipenem. Penicillinase degraded penicillin G most rapidly, followed by penicillin V and ampicillin. Oxacillin was very slowly degraded, while no detectable degradation of imipenem was observed. The penicillinase linear regression values for penicillin G, penicillin V, ampicillin, oxacillin and imipenem were 0.999, 0.999, 0.998, 0.867 and 0.324, respectively. MBL also showed the greatest activity against penicillin G and penicillin V. Degradation of ampicillin and imipenem was minimal (Fig. 5B), while no detectable degradation of oxacillin was observed. The MBL linear regression values for penicillin G, penicillin V, ampicillin, oxacillin and imipenem are 0.999, 0.998, 0.956, 0.377 and 0.909, respectively.

The calibration curves were very linear and highly reproducible (Fig. 5A and 5B). Our results were in accordance with their known specificity profiles (Citri, 1971; Pfeifer, *et al.*, 2010).

The FARA profiles for these two enzymes were visualized using two different schemes: one compared a single measurement for each antibiotic (Fig. 6A), while the other compared the calibration curve slopes (Fig. 6B). The 0.68 mM antibiotic concentration was chosen because it lies in the upper linear range which would be expected to highlight differences in enzyme activity, i.e. V_{max}, while the comparison of the slopes highlights differences in the steady state kinetics. The AR enzymes could easily be differentiated using both FARA data analysis schemes. The single data point scheme would provide the fastest assay cycle time, but may not provide enough information to identify the specific enzyme thus requiring additional data points to measured. We envision dynamic data analysis software, whereby data would be immediately analysed in order to determine if additional measurements were required to identify the specific enzyme. Given the large number β-lactamases and their close relationship, multiple antibiotic panels will be required. The availability of numerous antibiotics provides a nearly unlimited resource with which to develop targeted antibiotic panels. Ultimately, it should be possible to dynamically fine tune

the antibiotic panel as measurement results become available. Previously, we have used principle component analysis to identify patterns in complex data sets with excellent results (Mecklenburg *et al.*, 2002). Multivariate analysis techniques such as these will improve and accelerate AR diagnostics. Fast, accurate classification of β-lactamases will encourage the use of narrow spectrum antibiotics, which will reduce the use of broad spectrum antibiotics and the spread of AR.

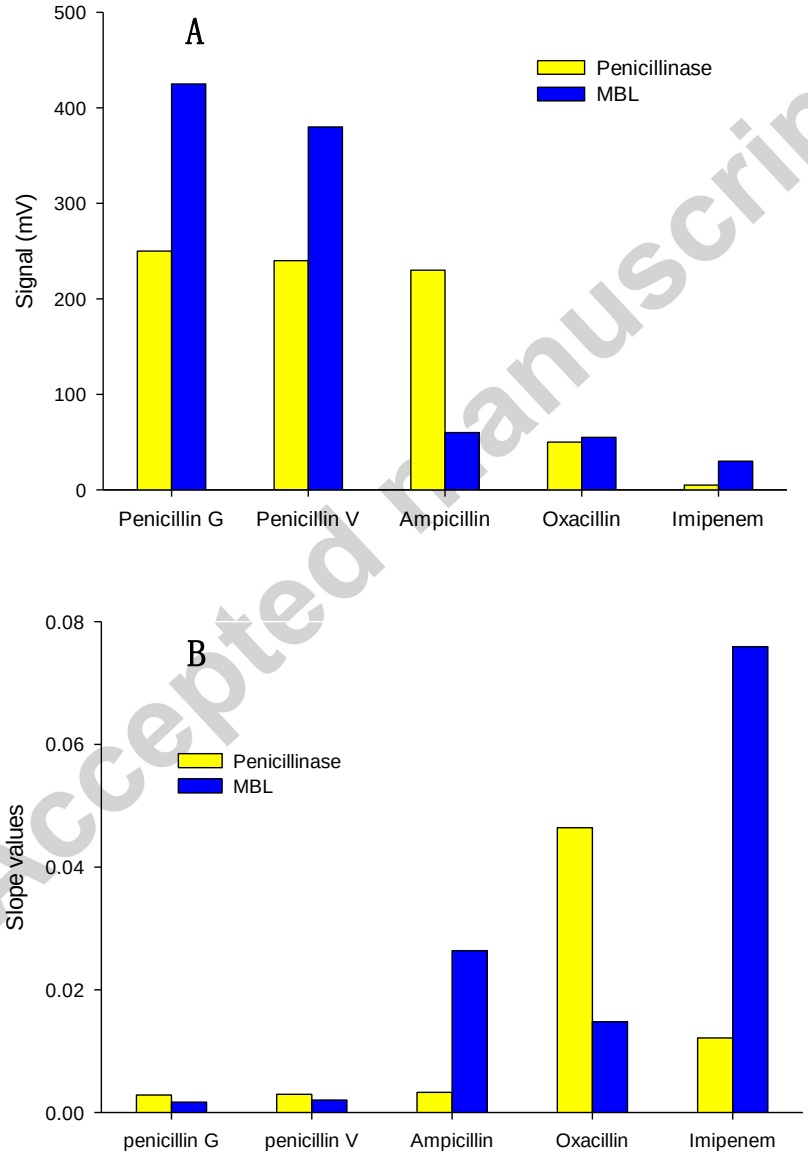

Fig. 6. Comparison of penicillinase and MBL antibiotic substrate specificity at a single antibiotic concentration of 0.68 mM (A) and slope calibration curves (B).

3.4 Broader implications of the technology

Previously we have developed a whole blood assay protocol which entirely eliminates the need for sample pretreatment (Chen *et al.*, 2013). Integrating this scheme and FARA opens up the possibility of developing an *in vitro* whole blood assay model that is capable of studying the interaction between AR enzymes and AR drugs in a native environment. One application of such a scheme is the analysis of antibiotic bioavailability (Merrikin *et al.*, 1983). A number of studies have shown that antibiotic binding to serum proteins is very common and yet no method for systematically analyzing these interactions *in vitro* is currently available (Zeitlinger *et al.*, 2011). Another potential *in vitro* assay application is the study of the effect that the various AR inactivator and inhibitor drugs cocktails have on AR enzymatic activity in blood. Systematic analysis of AR drug cocktails would expand our understanding of these interactions and could result in the development of entirely new therapeutic strategies (Chinwuba *et al.*, 1991; Kumar *et al.*, 2010).

4. Conclusions

The primary aim of this study was the development of a function-based assay capable of rapidly detecting and classifying AR. The FARA profiling strategy is based on the direct measurement of the thermal signal generated when AR enzymes catalytically degrade antibiotics. FARA was capable of differentiating two β-lactamase enzymes, penicillinase and MBL. Thermal activity profiling, combined with the availability of a wide range of antibiotics structures, provides us with a powerful tool with which to rapidly profile AR enzymes in about 1 hour. Profiling enhances the informational content of AR diagnostics which have the potential to revolutionize clinical diagnosis and treatments of AR infections. Future efforts will involve a retrospective analysis of clinical AR samples to determine the clinical utility of FARA. In addition, an *in vitro* whole blood model will be developed novel drug cocktails and

studying antibiotic interactions in the blood environment, i.e. bioavailability studies. Future studies will involve profiling AR enzymes known to be of clinical interest to determine if the these enzymes can be identified and tracked in the the clinical and environmental resistome.

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Highlights

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blood model for studying AR inhibitors and antibiotic availability is discussed.