USE OF THE RAPID CARBAPENEM INACTIVATION METHOD (RCIM) WITH CARBAPENEMASE INHIBITORS: A PROOF OF CONCEPT EXPERIMENT

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ABSTRACT

Introduction: One of the most worrying public health threat is the rapid dissemination of carbapenemase-producing Gram-negative bacteria, that are often multi-drug, if not pan-drug resistant leaving only very few therapeutic options for treating serious infections. Local testing is needed to support rapid detection and prompt action.

Objectives: To establish a protocol to detect the Ambler class of carbapenemases produced by *Enterobacteriaceae* using the Inhib-rCIM, an adaptation of the Rapid Carbapenem Inactivation Method (rCIM) in combination with class-specific inhibitors.

Methods: *Klebsiella pneumoniae* strains expressing various carbapenemases (KPC-1, NDM-1, OXA-48) and others lacking carbapenemase activity (*K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922) were incubated with Meropenem and Ambler class-specific inhibitors (phenylboronic acid, for class A and dipicolinic acid, for class B).

Results: Inhibition of class B carbapenemases was achieved starting with 16 μg/mL dipicolinic acid (DPA). Regarding phenylboronic acid (PBA), full inhibition of class A carbapenemases was not achieved even at 640 μg/mL of PBA, yet at two hours working time, the McFarland indices were nevertheless significantly different with that of the controls, permitting identification of carbapenemase class.

Conclusions: As novel antibiotic treatments based on inhibitor/beta-lactam combinations active only on class A and certain class D enzymes are available, rapid and cost effective detection methods used to identify the molecular class of the carbapenemase present are mandatory in order to curb with this problem. Inhib-rCIM is a rapid (less than 3 hours), cheap and easy-to-implement phenotypic test for the detection of Ambler classes of carbapenemases and could be useful especially in low resource settings.

Keywords: carbapenems, antimicrobial resistance, carbapenemase, inhibitors, *Enterobacteriaceae*, *Klebsiella pneumoniae*, rCIM

REZUMAT

Introducere: Unul din cele mai îngrijorătoare pericole din domeniul sănătății publice este reprezentat diseminarea rapidă a bacteriilor Gram-negative producătoare de carbapenemaze, ce sunt adesea multi-drog, dacă nu pan-drog rezistente. În aceste cazuri, opțiunile terapeutice sunt în număr foarte limitat în cazul infecțiilor severe. Testarea locală este necesară pentru a putea detecta rapid aceste organisme și a acționa prompt.

Obiective: Stabilirea unui protocol de detecție a clasei Ambler a carbapenemazelor produse de *Enterobacteriaceae* folosind Inhib-rCIM, o adaptare a Rapid Carbapenem Inactivation Method (rCIM) în combinație cu inhibitori specifici de clasă.

Metode: Tulpini de *Klebsiella pneumoniae* ce exprimă diverse carbapenemaze (KPC-1, NDM-1, OXA-48) și altele ce nu produc carbapenemaze (*K. pneumoniae* ATCC 700603 și *Escherichia coli* ATCC 25922) au fost incubate cu Meropenem și inhibitori specifici de clasă Ambler (acid fenilboronic pentru clasa A și acid dipicolinic pentru clasa B) pentru adaptarea protocolului rCIM.

Rezultate: Inhibiția carbapenemazelor de clasă B a fost obținută pornind de la o doză de 16 μg/mL acid dipicolinic (*dipicolinic acid*, DPA). În ceea ce privește acidul fenilboronic (*phenylboronic acid*, PBA), inhibiția totală a carbapenemazelor de clasă A nu a fost obținută nici la o concentrație de 640 μg/mL, putându-se evidenția nefelometric o creștere reziduală; totuși, la două ore de timp de lucru, indicele McFarland a prezentat o diferență semnificativă față de controale, permițând identificarea clasei Ambler.

Concluzii: Întrucât există noi tratamente disponibile bazate pe combinații de inhibitori/ß-lactamine active doar pe enzimele de clasă A și unele enzime de clasă D, este necesară dezvoltarea de noi metode de detecție a acestor clase. Inhib-rCIM este o metodă fenotipică rapidă (mai puțin de 3 ore), ieftină și ușor de implementat în orice laborator de microbiologie clinică, dar cu precădere în zonele cu resurse limitate.

Cuvinte-cheie: carbapeneme, rezistență antimicrobiană, carbapenemaze, inhibitori, *Enterobacteriaceae*, *Klebsiella pneumoniae*, rCIM

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INTRODUCTION

Carbapenemase-producing *Enterobacteriaceae* (CPE) represent a main public health threat as these bacteria are multi-, and sometimes pan-drug resistant [1].

There are increasing reports all over the world regarding outbreaks or even endemic situations with CPEs, both in the hospital setting and in the community, making their rapid detection a health priority [2].

Based on their primary structure, carbapenemases are either serine-beta-lactamases (Ambler classes A and D) or metallo-beta-lactamases (Ambler class B) [1]. Class A enzymes usually lack potent carbapenemase activity and primarily hydrolyze penicillins and cephalosporins; they are inhibited by a series of molecules such as clinically used beta-lactamase inhibitors (clavulanate, sulbactam, avibactam, relebactam) or boronic acid and its derivative, such as phenylboronic acid (PBA) [3]. The most frequently identified class A carbapenemases are KPC (*Klebsiella pneumoniae* carbapenemase), IMI, FRI-1, NMC-A, SME and some GES enzymes [4]. Class B enzymes, especially B1, which comprises the clinically most relevant enzymes, are capable of conferring high level of resistance to all beta-lactams, with the exception of aztreonam [1,3]. They are not inhibited by beta-lactamase inhibitors but instead, because their action is dependent on the presence of Zn^{2+} ions in their active site, they are inhibited by divalent cations chelators such as ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA) [1,5]. The most important members of the class B enzymes are NDM (New Delhi Metallo-beta-lactamase), VIM, IMP and SPM-1 [6,7]. Among Class D enzymes, also called oxacillinases or OXAs, OXA-48 is the most important carbapenemase. They hydrolyze carbapenems poorly and they are not inhibited by beta-lactam inhibitors nor EDTA but are inhibited by NaCl [1]. Attempts of using temocillin as an OXA-48 marker showed that it is not very specific, with reporting values being under 50% [8]. Currently, there are a number of available phenotypic tests using inhibitors such as the KPC/MBL & OXA-48 Confirm (KMOC) kit or the Mast Carbapenemase Detection Set™ [9,10], double-disk synergy tests using EDTA (Combined E-Tests) [11] that can be used in the microbiology laboratory for the detection of carbapenemases. These tests require an overnight incubation and display low specificities and sensitivities.

In this study we have developed the Inhib-rCIM for the detection of Ambler classes A, B and D using specific inhibitors.

MATERIALS AND METHODS

Bacterial isolates

Clinical isolates of carbapenem resistant *K. pneumoniae* were obtained from the Cantacuzino National Medico-Military Institute for Research and Development. Identification was done through the Vitek 2 system and MALDI-ToF. Carbapenemase activity was phenotypically confirmed through in-house Carba NP test [12]. The carbapenemase genes were identified by PCR and sequencing. Strains were chosen so that they harbored NDM-1, KPC-2, OXA-48, comprising one member of each Ambler class [13]. An SHV-18 expressing carbapenem-resistant reference *K. pneumoniae* strain with no carbapenemase activity was used as a negative control (ATCC 700603).

Inhib-rCIM

The protocol of the rCIM was previously published [14,15]. Briefly, two 10 µL loopfuls of an overnight culture of bacteria on a Trypticase Soy Agar (TSA) plate are suspended in 1000 µL of sterile water with 4 µg of Meropenem. For these preliminary, dose-ranging experiments, a carbapenemase inhibitor was added and the resulting suspension was incubated for 30 minutes. During this time, a growth indicator, consisting of *E. coli* ATCC 25922 at a McFarland Index of 1, was freshly prepared in Mueller-Hinton media. After centrifugation, 500 µL of supernatant was carefully taken and used for a subsequent antibiotic challenge, over 2500 µL of the growth indicator. The challenged growth indicator was monitored through nephelometry at 30 minute intervals, for at least 2 hours post challenge [15].

Continued, often unaffected, growth of the indicator strain implies that the Meropenem was inactivated, indicating the presence of carbapenemase activity. On the other hand, inhibition of the ATCC 25922 growth indicator

implies the persistence of the carbapenem and lack of carbapenemase activity. We showed this test to be highly sensitive and specific, even with enzymes that have an otherwise low hydrolyzing activity [15].

Carbapenemase classes were phenotypically determined through the use of two substances which act as carbapenemase class inhibitors: (i) Phenylboronic acid (PBA) which acts as an inhibitor of class A enzymes, such as KPC-2; (ii) Dipicolinic acid (DPA) which acts as an inhibitor of class B enzymes, such as NDM-1. They are no readily useful inhibitors which can be used for class D enzymes such as OXA-48, thus lack of inhibition with class A and B was taken as possible class D enzymes. Based on literature review, varying concentrations of inhibitors were used: 16-64 μg/mL of DPA and 160-640 μg/mL of PBA alone and in combination to assess for interaction.

Inhibition of the carbapenemases implies that the standard rCIM protocol detects carbapenemase activity, which would then be lost when a specific inhibitor is added. Thus, results were read and interpreted as pairs, using a cut-off of 0.5 McFarland of growth for the rCIM to evaluate the specific inhibitory effects of PBA and DPA on carbapenemases.

Statistical analysis

All experiments were carried out in triplicate. Results were recorded and imported into R (Version 3.4.2) with the addition of RStudio (Version 1.1.383) for analysis. Mann-Whitney's U test was used to compare the results. A linear regression was created for all carbapenemase producing and non-producing strains, with and without inhibitors.

RESULTS

Validation of the indicator strain growth curve and effect of inhibitors on growth curve

Growth controls consisted of *E. coli* ATCC 25922 resuspended to a McFarland's index of 1, challenged with sterile water. Negative growth controls were obtained by challenging the same bacteria using a suspension of 4 μg/mL of Meropenem. Growth of the indicator was similar as previously reported [16] (Fig. 1). Variability was small in this control set and growth is sufficiently well modeled through linear regression over such a short time course. At very high doses, DPA did not influence the growth curve of the ATCC 25922 *E. coli* indicator, having the same growth curve even at 64 μg/mL (4x) of DPA (data not shown).

While at 160 μg of PBA, there was no obvious effect on the *E. coli* indicator, a small, non-specific, inhibition of growth was evidenced with higher concentrations (320, 480 and 640 μg/mL) (Fig. 2).

Determination of concentration of Inhibition of PBA and DPA for carbapenemase activity inhibition

Dipicolinic acid is a specific inhibitor of metallo beta-lactams (class B carbapenemases, like NDM-1) (Fig. 3). Inhibition was achieved starting with 16 μg/mL, which is a concentration at which no intrinsic toxicity of DPA was observed.

Phenylboronic acid, an inhibitor of class A carbapenemases, such as KPC-2, showed a different inhibition pattern. An *a priori* starting concentration was chosen at 160 μg/mL, in accordance with the literature, but the response was suboptimal at this concentration and dose-dependent, with higher doses providing greater inhibition. Full inhibition was not achieved even at 640 μg/mL, with residual growth still being evidenced by nephelometry (25-33% of normal growth, representing a growth of about 0.4 to 0.5 McFarland units) (Fig. 4).

For cost-effectiveness, we considered that 320 μg/mL of PBA as being the best for determining the presence of specific inhibition of KPC-2. Inhibition, at this level, is roughly 50% of total growth, and, while this needs to be confirmed on other strains, seems reasonably easy to interpret. Nevertheless for ease of interpretation, a specific growth control with PBA should be included in order to evaluate the non-specific effect of PBA on the ATCC 25922 *E. coli* growth indicator strain (Fig. 5).

DISCUSSION

This study is a proof of concept and dose-ranging study of an adapted rCIM protocol, the Inhib-rCIM that can be used to phenotypically detect the class of carbapenemase produced by enterobacterial isolates in less than 3 hours. This test may be useful for clinical microbiology

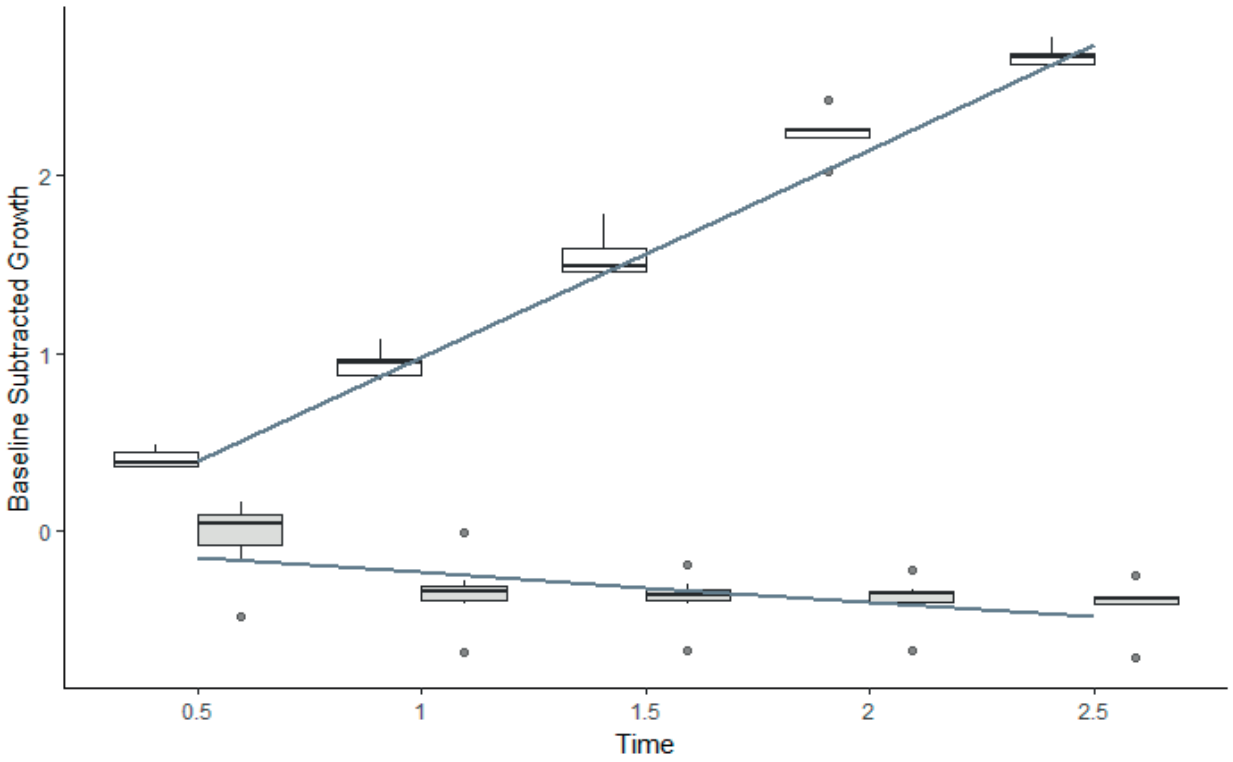

Fig. 1. Growth curve of positive and negative controls. Baseline subtracted growth was measured for two and a half hours after challenge. Boxplots show median values, quantiles and outliers. Linear regression models were drawn to fit the two growth models.

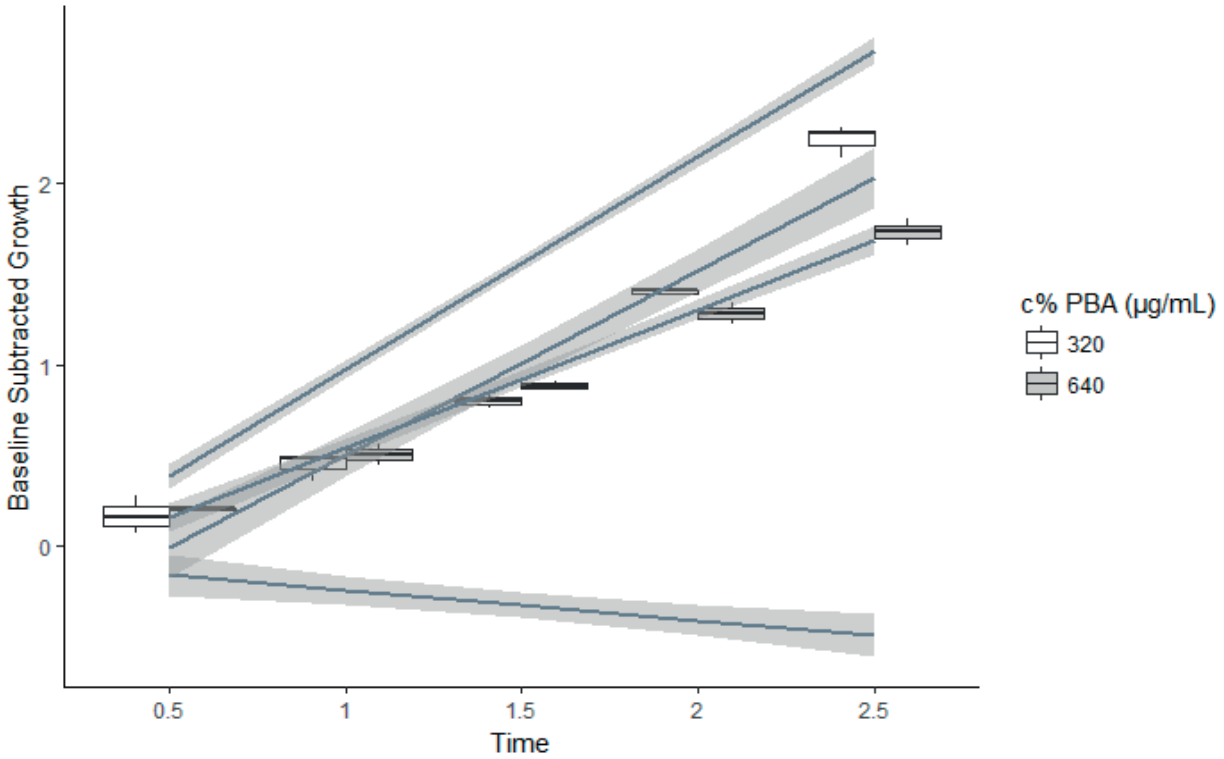

Fig. 2. Models for positive and negative control growth of *E. coli* ATCC 25922 with standard error (grey halo around the linear model). Growth curve of indicator strain showing inhibitory effect by nonspecific PBA activity. Baseline subtracted growth was measured for two and a half hours after challenge. Boxplots show median values, quantiles and outliers. Linear regression models were drawn to fit the two growth models.

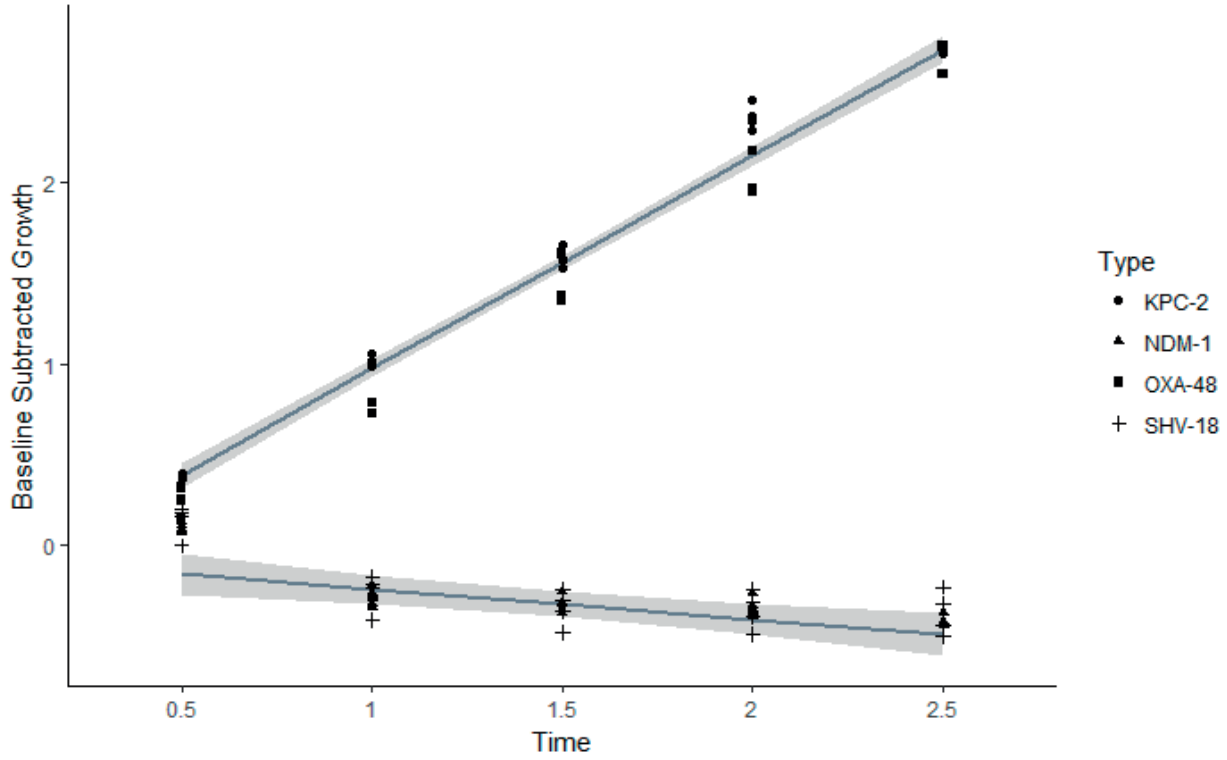

Fig. 3. Models for positive and negative control growth of *E. coli* ATCC 25922 with standard error. In the presence of its specific inhibitor (DPA), NDM-1 lost its carbapenemase activity, as the ATCC 25922 indicator's growth was completely inhibited. Different concentrations of DPA did not affect growth curves for the other carbapenemase producers. Baseline subtracted growth was measured for two and a half hours after challenge. Different shapes on the graph correspond to the different carbapenemase types produced by isolates. The SHV-18 producing strain showed normal rCIM response in the absence of a carbapenemase.

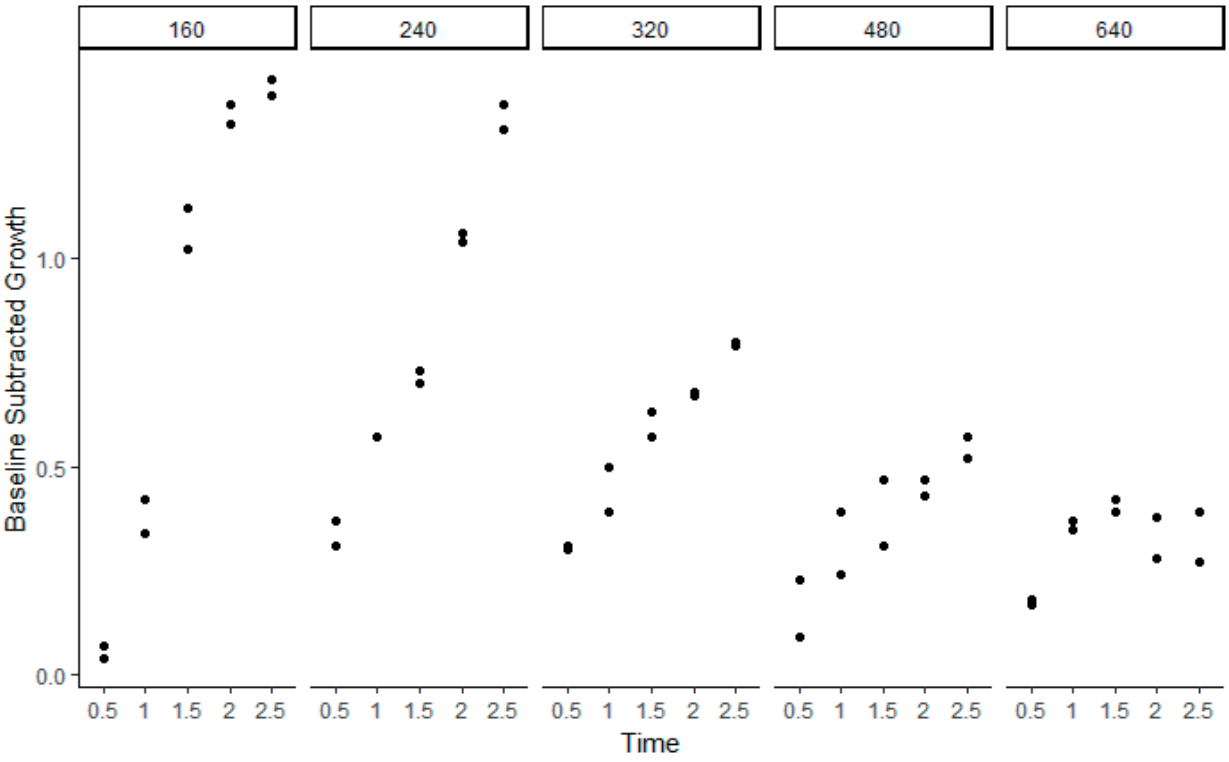

Fig. 4. Side-by-side breakdown of growth kinetics as a function of PBA concentration showing varying inhibition of KPC-2 activity. White rectangles represent the differing concentrations of PBA. Points represent baseline subtracted growth at each of the time points which are represented on the bottom axis.

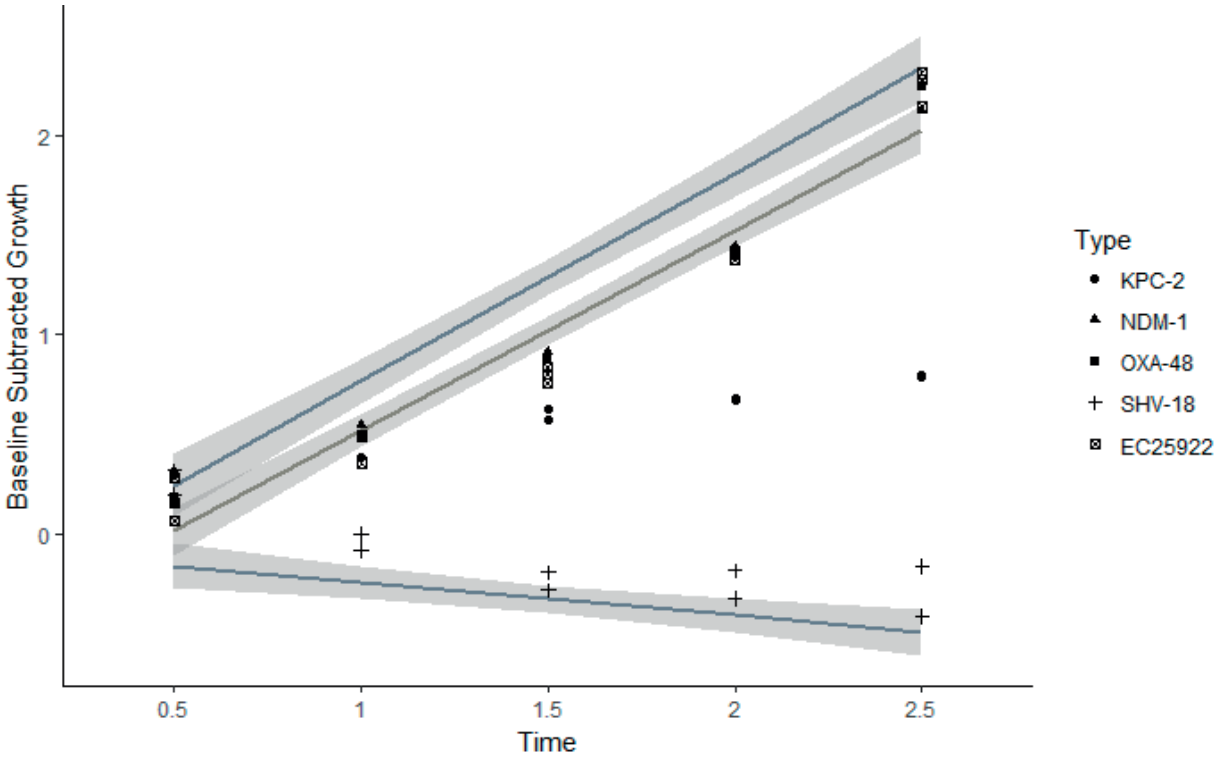

Fig. 5. Inhibitory activity of 320 µg/mL of PBA showing both specific (against KPC-2) and non-specific against (*E. coli* ATCC 25922 growth control and other carbapenemase-producers). Models for positive and negative control growth of *E. coli* ATCC 25922 with standard error as reference. The SHV-producing ATCC 700603 strain was not influenced.

laboratories, hygiene and infectious disease practitioners in multiple ways. Firstly, it is a rapid and cost-effective protocol that can be applied concomitantly or subsequently to the rCIM detection of carbapenemases. Secondly, it may have a cost-saving effect, as detection methods can be focused on carbapenemases of a specific class. Thirdly, it can help guide antibiotic therapy, as novel antibiotics and beta-lactamase inhibitors that act on specific classes of carbapenemases are being developed [17,18].

While Class A and Class B carbapenemases benefit from specific *in vitro* inhibitors, Class D does not have an inhibitor. We show that PBA has intrinsic toxicity on the *E. coli* indicator strain. Thus, even non-class A carbapenemase producers (like NDM-1, OXA-48) will have slightly reduced total growth. A growth control for which a PBA-challenge is applied is strongly advised to evaluate the growth curve. We believe that the dose of 320 µg/mL is the best compromise between specific and non-specific inhibition. DPA does not have any toxicity on the *E. coli* growth indicator. The main limitations of the study refer to the use of only the major carbapenemases (the 'big players'), from each group [19]. The most widely-spread carbapenemases called "the big five", which include the IMP and VIM metallo-beta-lactamases, which were not included in this study of *K. pneumoniae* isolates [20]. Another practical limitation is the need for a large quantity of bacteria (6 loopfuls) which may be difficult to obtain from one dispersion plate. As non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* pose a big clinical and epidemiological problem as well, studies are needed to validate the protocol. Finally, the usefulness of avibactam as inhibiting molecule, needs also to be evaluated.

Other methods and tests have been adapted to be used with carbapenemase inhibitors, like Combination disk test, Double disk synergy test and Gradient diffusion strips [21] and Rosco Neo-Sensitabs [9]. The main advantage of the Inhib-rCIM protocol over such techniques is the fast detection time (2 to 3 hours).

CONCLUSIONS

The study proposes a rapid (less than 3 hours) protocol for the phenotypic detection of carbapenemases belonging to the different Ambler classes. Similarly to the rCIM, the Inhib-rCIM, does not require trained personnel. The equipment needed is largely present in most microbiology laboratories, but the inhibitors must be purchased as they are not routinely available. Our study shows that the rCIM test can be adapted to be used with inhibitors, similarly to what has been achieved with biochemical tests [12]. Further studies are needed to validate this proof of concept protocol on clinical isolates and evaluate performance in clinical microbiology settings.

Conflict of interests: The authors have no conflict of interests to declare.

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