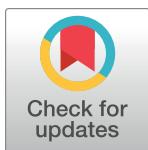


RESEARCH ARTICLE

Can flow cytometric measurements of reactive oxygen species levels determine minimal inhibitory concentrations and antibiotic susceptibility testing for *Acinetobacter baumannii*?



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Abstract

Current antimicrobial susceptibility testing (AST) requires 16–24 hours, delaying initiation of appropriate antibiotics. Hence, there is a need for rapid AST. This study aims to develop and evaluate the feasibility of a rapid flow cytometric AST assay to determine minimum inhibitory concentration (MIC) for carbapenem-resistant *Acinetobacter baumannii* (CRAB). Antibiotic exposure causes increased intracellular reactive oxygen species (ROS) in bacteria. We hypothesized that ROS can be used as a marker to determine MIC. We assessed three CRAB clinical isolates across fifteen antibiotics at various concentrations in a customized 96-well microtiter plate. The antibiotics assessed include amikacin, beta-lactams (ampicillin/sulbactam, aztreonam, cefepime, ceftolozane/tazobactam, doripenem, imipenem, meropenem, and piperacillin/tazobactam), levofloxacin, polymyxin B, rifampicin, trimethoprim/sulfamethoxazole, and tetracyclines (tigecycline and minocycline). These clinical CRAB isolates were assessed for ROS after antibiotic treatment. Increased ROS levels indicated by increased RedoxSensor™ Green (RSG) fluorescence intensity was assessed using flow cytometry (FCM). MIC was set as the lowest antibiotic concentration that gives a ≥ 1.5 -fold increase in mode RSG fluorescence intensity (MIC_{RSG}). Accuracy of MIC_{RSG} was determined by comparing against microtiter broth dilution method performed under CLSI guidelines. ROS was deemed accurate in determining the MICs for β -lactams (83.3% accuracy) and trimethoprim/sulfamethoxazole (100% accuracy). In contrast, ROS is less accurate in determining MICs for levofloxacin (33.3% accuracy), rifampicin (0% accuracy), amikacin (33.3% accuracy), and tetracyclines (33.3% accuracy). Collectively, this study described an FCM-AST assay to determine antibiotic susceptibility of CRAB isolates within 5 hours, reducing turnaround time up to 19 hours.

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Introduction

The *Acinetobacter baumannii* pathogen causes a range of life-threatening nosocomial infections [1]. The *A. baumannii* infections are associated with poor clinical outcomes, including attributable mortality rates of 8.4–36.5% [2] and excess 5–10 days of ICU stay [3,4]. Early treatment with appropriate antibiotics is vital to reduce mortality. Inappropriate antibiotics therapy can lead to increased risk of in-hospital mortality (adjusted relative risk ratio 1.76) [5] and increased 30-day mortality of 43.7% [6]. Delays in initiating appropriate antibiotics by ≥ 1 day is associated with 6.6% increase in mortality [7].

Appropriate treatment is further complicated by global increasing rates of resistance [8,9]. Multi-drug resistant (MDR) *A. baumannii* infection is associated with 5-fold increase in relative risk of receiving inappropriate empirical therapy [5]. Global investigation of multidrug-resistant *A. baumannii* revealed that resistance rates of imipenem (a carbapenem) increased exponentially every 5 years from 2000 to 2016 among countries in the Organization for Economic Cooperation and Development [10]. A study assessing *Acinetobacter* spp. collected from 140 hospitals across 32 countries revealed non-susceptibility to carbapenems doubled from 2005 to 2009 [11]. European surveillance data from 2009 showed that the *Acinetobacter* spp. was implicated in intensive care unit (ICU)-acquired pneumonia up to 21.8% of the time, in ICU-acquired bloodstream infections up to 17.1% and in ICU-acquired urinary tract infections up to 11.9% [12]. In Singapore, imipenem resistance rates in the largest tertiary acute care hospital, Singapore General Hospital in 2011–2015 was 4.3% higher compared to 2006–2008 [13]. Patients may only receive appropriate antibiotics after antimicrobial susceptibility testing (AST). Therefore, a short turn-around time to generate AST results is a crucial factor in ensuring early administration of appropriate antibiotics and successful treatment.

Current AST methods involve minimum inhibitory concentration (MIC) determination using culture-based methods, such as broth dilution and agar dilution assays. These culture methods are gold standards with high levels of sensitivity and specificity. However, current AST methods require 16–24 hours incubation following isolation of pure bacterial colonies [14,15]. This delays administration of appropriate antibiotics thereby increasing the likelihood of infection-related mortality. While rapid AST methods such as molecular and mass spectrometry-based methods were proposed and have been compared against the gold standards, these rapid AST methods may not always specify antibiotic susceptibility [16].

Flow cytometry (FCM) has been considered for rapid AST since the 1980s [17,18]. FCM can assess more than 10,000 cells per second. This rapid methodology will not only reduce the turnaround time significantly, delivering same-day results but is also able to detect bacteria sub-populations within the strains such as heteroresistant sub-populations. Currently, there is limited literature establishing a FCM assay that can be adopted for rapid AST covering a wide range of antibiotics [19–21]. Flow cytometry has been demonstrated to have good correlation to current automated diagnostic methods [22]. To the best of our knowledge, flow cytometric AST has yet to be established for routine clinical use globally.

Reactive oxygen species (ROS) is a collective term for all unstable, reactive oxygen-containing molecules. Exposing bacteria to antibiotics increases intracellular ROS, causing oxidative stress in bacteria [23–27]. Increased ROS can be simply measured and assessed by fluorometric methods such as flow cytometry. Therefore, we hypothesized that ROS is a suitable marker in predicting MIC and antibiotic susceptibility using FCM. Using ROS as an indicator, we aimed to evaluate the feasibility of an “one-protocol-fits-all-antibiotics” FCM-AST assay.

Materials and methods

Bacteria isolates

Nonclonal clinical strains of CRAB previously collected from the largest tertiary hospital in Singapore as part of a nationwide surveillance study from 2009 to 2016, were used in this study. Genus identity was determined using Vitek 2 ID-GN cards (bioMérieux, Inc., Hazelwood, MO). Bacteria isolates were stored at -80°C. Isolates were sub-cultured twice on Tripticase Soy Agar (TSA) with 5% sheep blood (Thermo Fisher, Singapore) at 35°C before each experiment. Isolates were randomly selected for this study. Three CRAB clinical isolates (AB0047, AB0356, AB0603) were used in this study (S1 Table in [S1 File](#)). The lab adapted ATCC19606 strain was used as a reference strain for characterizing the RSG fluorophore.

Fluorophores used for FCM

The fluorescent dye, RedoxSensor™ Green (RSG) of the *BacLight* RedoxSensor Green Vitality Kit (Thermo Fisher Scientific, Singapore), was used to assess bacterial ROS. Increased RSG fluorescence intensity indicates increased intracellular ROS. To identify viable bacteria for ROS assessments, the fluorescent dyes, SYTO-62 and propidium iodide (PI), (Thermo Fisher Scientific, Singapore) were used together with RSG. SYTO-62 labels nucleic acid of all bacteria. PI only enters non-viable bacteria with compromised membranes and intercalate DNA.

Characterization of RSG fluorophore

The ATCC 19606 *A. baumannii* reference strain was used to characterize the RSG fluorophore. An inoculum of $10^{5.7}$ bacteria were stained with 20 μ M RSG at room temperature for 15 mins. Stained bacteria were then exposed to various ROS-generating reagents. Hydrogen peroxide and sodium hypochlorite were added at a final concentration of 10 mM and 1.5 mM respectively. Hydroxyl radicals were generated using 1mM ferrous sulfate heptahydrate reacting with 200 μ M hydrogen peroxide [28]. Samples were then assessed by FCM at every timepoint.

FCM assessments of MIC

Similar to standard microtiter broth dilution method, bacteria suspension at $10^{5.7}$ CFU/mL was inoculated into each well of a customized microtiter broth dilution panel. The plate was incubated at 35°C for 30 minutes. Bacteria were then stained with the fluorescence cocktail (20 μ M RSG, 1 μ M SYTO-62 and 20 μ M PI) and incubated for a further 15 minutes at room temperature before FCM assessments. Data acquisition was carried out with CytoFlex (Beckman Coulter, Singapore). Details on instrumental settings and configurations during data acquisition can be found in (S2 Table in [S1 File](#)). The manufacturer settings with a default “medium” flow rate of 30 μ L/min was used. Each well was sampled for 180 seconds or until 20,000 events were collected, whichever came first. The durations for sample mixing and back-flush of the fluidic system were set at 5 s and 60 s respectively to ensure accuracy of FCM assessments. Data acquired were exported in .fcs files and analyzed using the FlowJo software (v10.4, Tree Star, Ashland, OR, USA). Gating strategy and compensation matrix can be found in the (S1 Fig in [S1 File](#) and S2 Table in [S1 File](#)).

Standard microtiter broth dilution method

MIC was determined using standard microtiter broth dilution method with customized 96-well microtiter broth dilution panels (Trek Diagnostics, East Grinstead, UK) performed under CLSI guidelines. Briefly, bacterial suspension of $10^{7.7}$ CFU/mL in physiological saline (0.85% (w/v) sodium chloride) was prepared. Ten-fold dilutions were performed to achieve

$10^{5.7}$ CFU/mL in cation-adjusted Müller-Hinton broth. Microtiter broth dilution panel was inoculated with 100 μ L of bacterial suspension at $10^{5.7}$ CFU/mL per well. Bacterial growth is assessed after the microtiter plate was then incubated at 35°C over 20–24 hours. Susceptibility breakpoints from MICs were interpreted primarily based on CLSI guidelines. Breakpoints from other sources were used when the breakpoint is not indicated in CLSI.

Results

Establishing optimal conditions for RedoxSensor Green to measure intracellular ROS

The commercial RedoxSensor™ Green (RSG) fluorophore can assess bacterial intracellular redox environment [29]. The RSG probe was used to sort dormant bacteria known as persisters, based on intracellular redox environment [30,31]. We first established optimal conditions for RSG to detect specific intracellular ROS.

To minimize bacterial redox buffering response to the ROS-generating reagents, the ATCC 19606 reference strain was first stained with RSG before exposing to ROS-generating reagents. Bacteria were then assessed using FCM. The RSG mode fluorescence intensities (peak of histogram) obtained from flow cytometric analyses were then compared against untreated conditions (Fig 1; panel A).

As recommended by the consensus established, we developed our FCM assay with an attempt to define the specific ROS measured by RSG [32]. Both hydrogen peroxide (H_2O_2) and hypochlorite ($HOCl$) revealed an increase in RSG mode fluorescence intensity (MFI) compared to untreated bacteria at 45 minutes (Fig 1; panel B). Alterations in metabolism associated with oxidative damage are present as short as 30 minutes of antibiotics exposure. [33]. Therefore, in selecting a shorter antibiotic exposure time, we anticipate an increase in viability of bacteria for better ROS assessments. Hence, 45 minutes was used for FCM measurements of

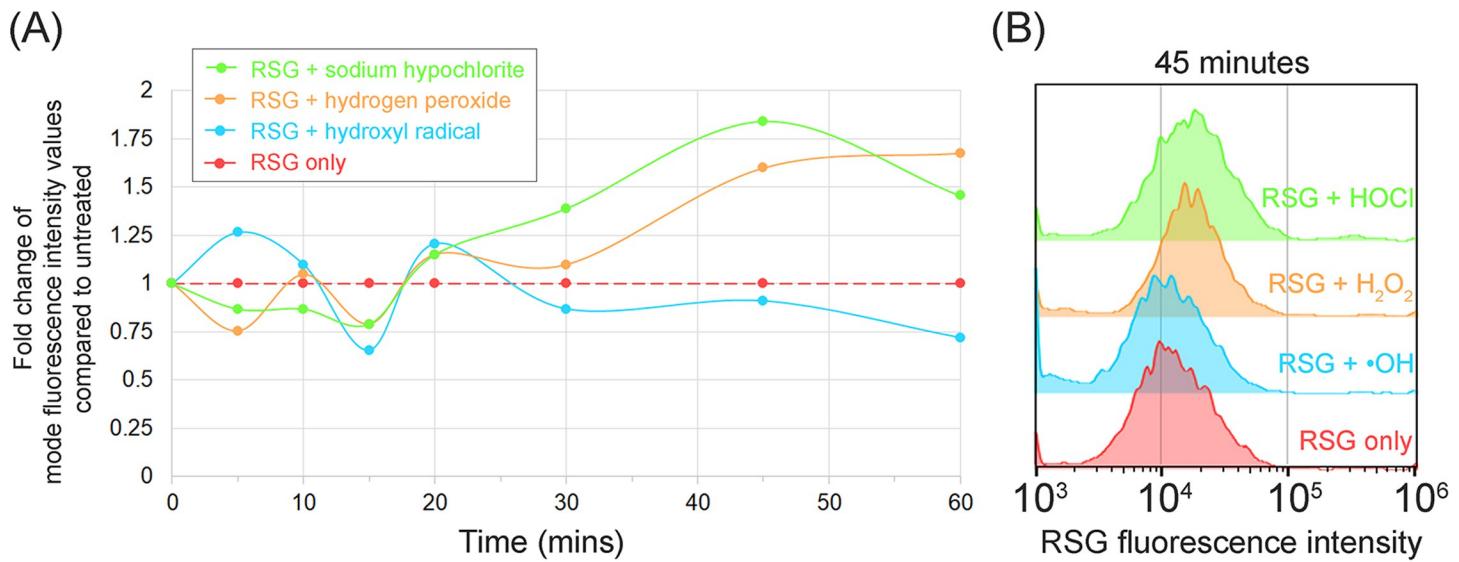


Fig 1. Establishing a FCM assay by characterising RSG for assessing oxidative stress in antibiotic exposed *A. baumannii*. The lab adapted strain, ATCC 19606, was first stained with RSG, before exposing to the ROS-generating reagents at room temperature. Bacteria were then assessed by FCM. Mode fluorescence intensities obtained from flow cytometric analyses were compared against untreated conditions as shown in panel (A). Flow cytometric histograms revealing the optimal fold change of RSG fluorescence intensities at 45 minutes were shown in panel (B).

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antibiotic-treated bacteria in this study. The ≥ 1.5 -fold increase observed was also used as the minimum cut-off for increased ROS in subsequent experiments.

Flow cytometric AST assay to predict MIC using ROS as a marker

We next investigated ROS as a marker in our assay to predict MIC. Using a cocktail of various fluorophores, we assessed the oxidative stress in viable bacteria after exposure to antibiotics (Fig 2; Panel A). This fluorophore cocktail consists of RSG, SYTO-62 and propidium iodide (PI). Viable bacteria are defined as bacteria stained with SYTO-62 but not PI.

Bacteria strains were first exposed to varying concentrations of antibiotics in a customised microtiter panel for 30 minutes. These antibiotics include amikacin, ampicillin/sulbactam, aztreonam, cefepime, cefotolozane/tazobactam, doripenem, imipenem, levofloxacin, meropenem, minocycline, piperacillin/tazobactam, polymyxin-B, rifampicin, tigecycline and trimethoprim/sulfamethoxazole. Without removing the antibiotics, bacteria were then stained with the fluorescence cocktail for a further 15 minutes at room temperature. This staining protocol was adapted from protocol suggested by manufacturers, using metabolic inhibitors.

Flow cytometric analyses revealed increased RSG fluorescence intensities of viable bacteria populations after exposure to several antibiotics (Fig 2; Panel B). Increased RSG fluorescence intensities indicate increased ROS levels, supporting the basis that antibiotics exposure increases ROS production [23–27].

To avoid bias from human curation in determining MIC, we opted an arithmetic approach in determining MIC from the flow cytometry plots. Mode values of RSG fluorescence intensities (i.e., peak of histograms) at each drug concentration were compared against untreated controls (Fig 2). Adapting from our RSG characterization studies (Fig 1), a ≥ 1.5 -fold increase in RSG fluorescence indicates an increase in bacterial ROS. Sub-inhibitory concentrations of antibiotics will result in negligible oxidative stress [34]. Therefore, the MIC was determined from the lowest antibiotic concentration that gives a ≥ 1.5 -fold increase in mode RSG fluorescence intensity (MIC_{RSG}).

A summary of these MICs determined from RSG fluorescence assessed by flow cytometry is reflected in Table 1. As the viable bacteria exposed to polymyxin-B remains very low (< 10%), we are unable to determine the MICs for polymyxin-B.

Comparison of the methods: FCM AST against standard microtiter broth dilution method

To determine the accuracy of our FCM AST assay, the MICs of antibiotics determined by FCM were compared against the standard microtiter broth dilution method (Table 1). MICs determined by FCM that are within ± 2 -fold dilutions of the MIC determined by microtiter broth dilution was deemed as accurate.

Flow cytometric determination of MICs using ROS as a marker was deemed accurate for β -lactams (83.3% accuracy) and trimethoprim/sulfamethoxazole (100% accuracy) as compared to the standard microtiter broth dilution method. In contrast, FCM is less accurate in determining the MICs for levofloxacin (33.3% accuracy), rifampicin (0% accuracy), amikacin (33.3% accuracy), and tetracyclines (33.3% accuracy) when using ROS as a marker.

We then further verify if the MICs predicted by the 2 different methods affects the antibiotic susceptibilities breakpoints (Tables 1 and 2). The susceptibilities breakpoints (i.e., susceptible/intermediate/resistant) were primarily inferred from the CLSI guidelines. The EUCAST or recommended breakpoints by literature [35] were used when susceptibility breakpoints were not available on CLSI. We observed that the susceptibilities profile remains $>95\%$ accurate for β -Lactams, levofloxacin, rifampicin, and trimethoprim/sulfamethoxazole. In other

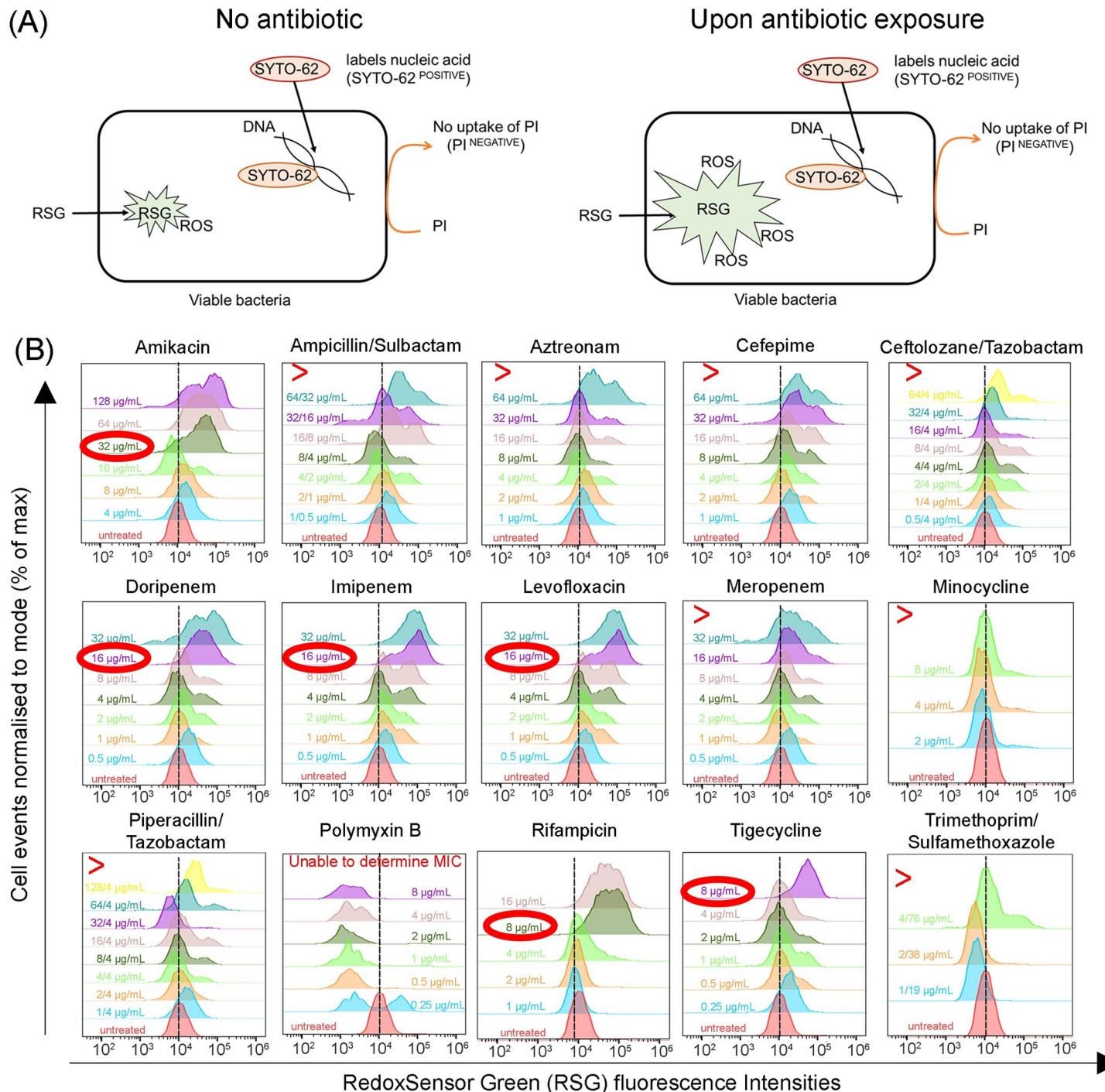


Fig 2. RSG fluorescence intensities in bacteria changes upon antibiotics exposure. Panel (A) describes the labelling strategy to assess ROS in viable bacteria using a cocktail of dyes. SYTO-62 labels all bacteria by binding to nucleic acid, while propidium iodide (PI) only enters non-viable bacteria. Viable bacteria (takes up SYTO-62 but not PI) will be assessed for ROS. Basal ROS is generated as a by-product of respiration under homeostatic conditions, hence exhibiting RSG fluorescence. After bacteria were exposed to antibiotics, there will be an increase in ROS. This increase in intracellular ROS, increases the intensity of RSG fluorescence measurable by flow cytometry. (B) Representative flow cytometric histograms depicting the changes in RSG fluorescence intensities in clinical isolate (AB0047) upon exposure to various antibiotics. Flow cytometric histograms for clinical isolates (AB0356 and AB0603) are shown in S2 and S3 Figs in S1 File. Exposure to sufficient concentrations of antibiotics resulted in increased RSG fluorescence as indicated by rightward shifts of the histograms. The antibiotic concentration corresponding to the first histogram shift compared to untreated (no antibiotics) was determined to be the MIC of that antibiotic (circled in red). The '>' symbol indicates MIC is higher than the highest concentration tested in the customized microtiter panel.

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Table 1. Antibiotics susceptibility profiles of clinical AB isolates.

MIC (mg/L)	AB0047		AB0356		AB0603		MIC _{RSG}
Antibiotics	MIC _{BMD}	MIC _{RSG}	MIC _{BMD}	MIC _{RSG}	MIC _{BMD}	MIC _{BMD}	
AMK	≥256 (R)	32 (I)	≤ 4 (S)	≤ 4 (S)	≥256 (R)	≥256 (R)	≥256 (R)
ATM ¹	≥128 (R)	≥128 (R)	≥128 (R)	64 (R)	64 (R)	≥128 (R)	≥128 (R)
C/T ²	≥128/4 (R)	≥128/4 (R)	≥128/4 (R)	1/0.5 (S)	64/4 (R)	≥128/4 (R)	≥128/4 (R)
DOR	32 (R)	32 (R)	32 (R)	1 (S)	32 (R)	≥64 (R)	≥64 (R)
FEP	≥128 (R)	≥128 (R)	64 (R)	≥128 (R)	≥128 (R)	≥128 (R)	≥128 (R)
IPM	≥64 (R)	32 (R)	16 (R)	32 (R)	32 (R)	≥64 (R)	≥64 (R)
LVX	32 (R)	16 (R)	16 (R)	>32 (R)	32 (R)	≥64 (R)	≥64 (R)
MER	≥64 (R)	≥64 (R)	32 (R)	1 (S)	32 (R)	≥64 (R)	≥64 (R)
MIN	4 (S)	≥16 (R)	≤ 2 (S)	4 (S)	8 (I)	≥16 (R)	≥16 (R)
PMB	0.5 (I)	Unable to determine	0.5 (I)	Unable to determine	0.5 (I)	0.5 (I)	0.5 (I)
RIF ³	≥32 (R)	≥32 (R)	4 (R)	≥32 (R)	4 (R)	≥32 (R)	≥32 (R)
SAM	32/16 (R)	≥128/64 (R)	32/16 (R)	1/0.5 (S)	≥128/64 (R)	≥128/64 (R)	≥128/64 (R)
SXT	≥8/152 (R)	≥8/152 (R)	≥8/152 (R)	≥8/152 (R)	≥8/152 (R)	≥8/152 (R)	≥8/152 (R)
TGC ⁴	2 (S)	8 (R)	1 (S)	0.5 (S)	1 (S)	≥16 (R)	≥16 (R)
TZP	≥256/4 (R)	≥256/4 (R)	≥256/4 (R)	128/4 (R)	≥256/4 (R)	≥256/4 (R)	≥256/4 (R)
Inoculum	10 ^{5.76}	10 ^{5.83}	10 ^{6.13}	10 ^{5.85}	10 ^{5.92}	10 ^{6.01}	

Table shows MICs of the three clinical CRAB isolates (AB0047, AB0356, AB0603). MICs obtained were interpreted based on CLSI guidelines, otherwise stated. Breakpoints against aztreonam, ceftolozane/tazobactam, rifampicin and tigecycline against AB were determined using breakpoints stated in literature. MICs for respective antibiotic as determined by both techniques, found within 1-fold dilution are deemed as a “hit” and are indicated in **bold**. Abbreviations: MIC_{BMD}: Minimal inhibitory concentrations determined from standard microtiter broth dilution; MIC_{RSG}: Minimal inhibitory concentrations determined from RSG mode fluorescence intensity fold differences; (S): Susceptible; (I): Intermediate; (R): Resistant; AMK: Amikacin; ATM: Aztreonam; C/T: Ceftolozane/Tazobactam; DOR: Doripenem; FEP: Cefepime; IPM: Imipenem; LVX: Levofloxacin; MER: Meropenem; MIN: Minocycline; PMB: Polymyxin B; RIF: Rifampicin; SAM: Ampicillin/Sulbactam; SXT: Trimethoprim/Sulfamethoxazole; TGC: Tigecycline; TZP: Piperacillin/Tazobactam; ¹Breakpoints based on EUCAST *P. aeruginosa* breakpoints; ²Breakpoints for AB determined as: Susceptible ≤ 2/4 µg/mL; Resistant: ≥ 4/4 µg/mL [35]; ³Breakpoints based on CLSI *Staphylococcus* breakpoints; ⁴Breakpoints based on FDA *Enterobacteriaceae* breakpoints.

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Table 2. Summary of MICs comparisons between standard microtiter broth dilution method and MICs determined via FCM (using ROS as a marker).

Antibiotics	MICs within ± 2-fold dilution	Matched antibiotic susceptibility breakpoints
Amikacin	1/3 (33.3%)	1/3 (33.3%)
β-lactams	20/24 (83.3%)	23/24 (95.8%)
Levofloxacin	1/3 (33.3%)	3/3 (100%)
Rifampicin	0%	3/3 (33.3%)
Tetracyclines	2/6 (33.3%)	1/6 (33.3%)
Trimethoprim/sulfamethoxazole	3/3 (100%)	3/3 (100%)
Polymyxin B [#]	1/1 (100%)	1/1 (100%)

Antibiotics of which the MICs are compared between the FCM and standard microtiter broth dilution method as grouped and shown in the first column. MICs determined from FCM analyses that falls within ±2-fold dilution of the MICs from standard microtiter broth dilution method were indicated as accurate in the second column. MICs predicted from both methods were interpreted using CLSI guidelines and the breakpoints were compared. Matched breakpoints were indicated as accurate and shown in the last column. [#]Unable to determine MIC of polymyxin B for AB0047 and AB0356 strains due to very low viable cells (<10%) available for flow cytometric assessments of ROS.

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words, the MICs from the two techniques were still giving similar antibiotic susceptibility breakpoints for β -Lactams, levofloxacin, rifampicin, and trimethoprim/sulfamethoxazole. The susceptibilities profile for amikacin (33.3% accuracy) and the tetracyclines (16.7% accuracy) were vastly different when inferring from MICs of the two techniques. This implies that the antibiotic susceptibilities inferred from the MICs of the two techniques were different for amikacin and tetracyclines

Discussion

The *A.baumannii* is an aerobic pathogen that causes a range of life-threatening nosocomial infections with poor clinical outcomes [1]. Early treatment with appropriate antibiotics is vital to reduce mortality. Thus, time taken to produce AST results is a crucial factor in ensuring early administration of appropriate antibiotics and successful treatment.

One major advantage of FCM is the rapid assessments of cells at the single cell resolution. FCM assay also offers an advantage in higher flexibility compared to current automated AST tools, such as Vitek-2 [22,36]. High correlations (>90%) of flow cytometric determination of AST against Vitek-2 was documented across multiple species [22]. These flexibilities include testing any bacteria species against any antibiotics, including antibiotics combinations [37–39].

A major pitfall of FCM-based assays for routine use is data analyses. FCM data analyses are often time-consuming, requiring high technical expertise and can be subjective with high user variability. To improve reproducibility and reduce user variability, we adopted auto-contouring gating (S2 Fig in [S1 File](#)). We envisioned an automated workflow from the extractions of mode fluorescence intensities and subsequent comparison to a single MIC value output without human intervention [40].

Fluorescence methodologies, such as FCM, remain one of the simplest measurements of ROS in live cells. ROS arises from basal metabolism as a by-product of electron transport chain [25]. Exposing bacteria to antibiotic activates stress response pathways leading to a higher increase of intracellular ROS [23–27]. In *A. baumannii*, polymyxins increase hydroxyl radicals [41], which can be further amplified with rifampicin [42]. Ajiboye and colleagues demonstrated increased superoxide ion generation, NAD^+/NADH ratio and ADP/ATP ratio in *A. baumannii* when exposed to various antibiotics at 4X higher MIC [43–47]. Dwyer and colleagues demonstrated higher hydrogen peroxide (H_2O_2) levels with ampicillin exposure compared to norfloxacin, while superoxide ($\bullet\text{O}_2^-$) levels remain similar for both antibiotics [23]. This suggests that specific ROS might be generated in bacteria, in response to different antibiotics. However, we are unable to discern the specific ROS for each antibiotic through our assay.

We reasoned that the inaccuracies in our assay stemmed from the different mechanisms of actions of individual antibiotics. Aminoglycosides are broad-spectrum antibiotics that inhibit protein synthesis, by primarily binding to the aminoacyl site of 16S ribosomal RNA within the 30S ribosomal subunit. Similar to our observation for amikacin in clinical isolates, multiple aminoglycosides at concentrations higher than MICs did not generate ROS in lab-adapted *A. baumannii* strains [48]. Polymyxin B binds and neutralizes the lipopolysaccharide of Gram-negative bacteria, disrupting membrane integrity. Due to the pharmacokinetics of polymyxin-B, 45 minutes might not be the optimal duration to assess viable cells for polymyxin-B induced oxidative stress. Decreased oxidative respiration were reported in *Escherichia coli* and *Staphylococcus aureus* treated with “bacteriostatic” antibiotics, such as rifampicin that inhibits bacterial RNA polymerase, tetracyclines that inhibit protein synthesis via inhibition of 30s ribosome and levofloxacin that inhibit DNA synthesis [49]. Consistent to our data, these antibiotics do not reveal an increase in RSG fluorescence intensity.

Literature had debated if ROS is the key mediator of antibiotic bactericidal effect [34,50,51]. This current study neither contests nor concurs with the association of ROS to be the cause of bactericidal effects of antibiotics. This study also does not dissect the mechanisms of antibiotics actions nor the bacterial redox defense mechanisms against antibiotics. Rather, this study builds on the foundation that by exposing bacteria to a stress stimulus, such as sufficient concentrations of antibiotics, ROS will be increased in bacteria causing oxidative stress.

We envisaged that measuring bacterial ROS can also be further used to detect heteroresistant sub-populations routinely. There is increasing evidence that heteroresistance can lead to treatment failure and detecting heteroresistance is crucial for appropriate antibiotics for a successful treatment outcome. [52,53]. Current gold-standard in determining heteroresistance is population analysis profiling, which is tedious and laborious for routine clinical laboratory diagnosis [54–56]. Single-cell assessments by FCM can detect heterogenous sub-populations rapidly. Isogenic resistant sub-populations have lower oxidative burst than the susceptible counterparts upon antibiotic exposure [57]. Hence, our protocol will reveal a multi-modal histogram when assessing RSG fluorescence.

Further studies will aim to optimize this setup in attaining our aim for an “one-protocol-fits-all-antibiotics” FCM-AST workflow for *A. baumannii*. Nevertheless, this current protocol still demonstrates that ROS is a good indicator for determining MIC for β -lactams and Trimethoprim/sulfamethoxazole.

In summary, this study described the workflow of a rapid FCM-AST assay to determine antibiotic susceptibility of CRAB isolates against fifteen different antibiotics. We demonstrate that ROS is a good indicator for determining MICs for β -lactams and Trimethoprim/sulfamethoxazole. The workflow can produce results within 5 hours, up to 19 hours earlier compared to standard AST methods. Our assay can potentially translate to faster initiation of appropriate antibiotics, and hence, improved clinical outcomes.

Supporting information

S1 File. All supplementary tables and figures can be found in the supporting information file “Yeo et al Supporting information.”.

(DOCX)

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Writing – review & editing: Jia Hao Yeo, Andrea Lay-Hoon Kwa.

References

1. Moubareck CA, Halat DH. Insights into *Acinetobacter baumannii*: A review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen. *Antibiotics*. 2020;9. <https://doi.org/10.3390/antibiotics9030119> PMID: 32178356
2. Falagas ME, Rafaillidis PI. Attributable mortality of *Acinetobacter baumannii*: No longer a controversial issue. *Crit Care*. 2007; 11: 11–13. <https://doi.org/10.1186/cc5911> PMID: 17543135
3. Lee H, Lee H. Clinical and economic evaluation of multidrug-resistant *Acinetobacter baumannii* colonization in the intensive care unit. *Infect Chemother*. 2016; 48: 174–180. <https://doi.org/10.3947/ic.2016.48.3.174> PMID: 27659440
4. Sileem AE, Said AM, Meleha MS. *Acinetobacter baumannii* in ICU patients: A prospective study highlighting their incidence, antibiotic sensitivity pattern and impact on ICU stay and mortality. *Egypt J Chest Dis Tuberc*. 2017; 66: 693–698. <https://doi.org/10.1016/j.ejcdt.2017.01.003>
5. Zilberberg MD, Nathanson BH, Sulham K, Fan W, Shorr AF. Multidrug resistance, inappropriate empiric therapy, and hospital mortality in *Acinetobacter baumannii* pneumonia and sepsis. *Crit Care*. 2016; 20: 1–10. <https://doi.org/10.1186/s13054-016-1392-4> PMID: 27417949
6. Kwon KT, Oh WS, Song JH, Chang HH, Jung SI, Kim SW, et al. Impact of imipenem resistance on mortality in patients with *Acinetobacter* bacteraemia. *J Antimicrob Chemother*. 2007; 59: 525–530. <https://doi.org/10.1093/jac/dkl499> PMID: 17213265
7. Lim C, Mo Y, Teparrukkul P, Hongswan M, Day NPJ, Limmathurotsakul D, et al. Effect of Delays in Concordant Antibiotic Treatment on Mortality in Patients With Hospital-Acquired *Acinetobacter* Species Bacteremia: Emulating a Target Randomized Trial With a 13-Year Retrospective Cohort. *Am J Epidemiol*. 2021; 190: 2395–2404. <https://doi.org/10.1093/aje/kwab158> PMID: 34048554
8. Mera RM, Miller LA, Amrine-Madsen H, Sahm DF. *Acinetobacter baumannii* 2002–2008: Increase of carbapenem-associated multiclass resistance in the United States. *Microb Drug Resist*. 2010; 16: 209–215. <https://doi.org/10.1089/mdr.2010.0052> PMID: 20707714
9. Hsu L-Y, Apisarnthanarak A, Khan E, Suwantararat N, Ghafur A, Tambyah PA. Carbapenem-Resistant *Acinetobacter baumannii* and Enterobacteriaceae in South and Southeast Asia. *Clin Microbiol Rev*. 2017; 30: 1–22. <https://doi.org/10.1128/CMR.masthead.30-1> PMID: 27795305
10. Lee YL, Ko WC, Hsueh PR. Geographic patterns of *Acinetobacter baumannii* and carbapenem resistance in the Asia-Pacific Region: results from the Antimicrobial Testing Leadership and Surveillance (ATLAS) program, 2012–2019. *Int J Infect Dis*. 2023; 127: 48–55. <https://doi.org/10.1016/j.ijid.2022.12.010> PMID: 36516915
11. Mendes RE, Farrell DJ, Sader HS, Jones RN. Comprehensive assessment of tigecycline activity tested against a worldwide collection of *Acinetobacter* spp. (2005–2009). *Diagn Microbiol Infect Dis*. 2010; 68: 307–311. <https://doi.org/10.1016/j.diagmicrobio.2010.07.003> PMID: 20955916
12. Abbott I, Cerqueira GM, Bhuiyan S, Peleg AY. Carbapenem resistance in *Acinetobacter baumannii*: Laboratory challenges, mechanistic insights and therapeutic strategies. *Expert Rev Anti Infect Ther*. 2013; 11: 395–409. <https://doi.org/10.1586/eri.13.21> PMID: 23566149
13. Teo JQM, Cai Y, Lim T-PP, Tan TT, Kwa ALH. Carbapenem resistance in gram-negative bacteria: The not-so-little problem in the little red dot. *Microorganisms*. 2016; 4: 1–11. <https://doi.org/10.3390/microorganisms4010013> PMID: 27681907
14. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc*. 2008; 3: 163–175. <https://doi.org/10.1038/nprot.2007.521> PMID: 18274517
15. Syal K, Mo M, Yu H, Iriya R, Jing W, Guodong S, et al. Current and emerging techniques for antibiotic susceptibility tests. *Theranostics*. 2017; 7: 1795–1805. <https://doi.org/10.7150/thno.19217> PMID: 28638468
16. Rentschler S, Kaiser L, Deigner HP. Emerging options for the diagnosis of bacterial infections and the characterization of antimicrobial resistance. *Int J Mol Sci*. 2021; 22: 1–29. <https://doi.org/10.3390/ijms22010456> PMID: 33466437

17. Martinez O V., Gratzner HG, Malinin TI, Ingram M. The effect of some β -lactam antibiotics on *Escherichia coli* studied by flow cytometry. *Cytometry*. 1982; 3: 129–133. <https://doi.org/10.1002/cyto.990030211> PMID: 6216082
18. Steen HB, Boye E, Skarstad K, Bloom B, Godal T, Mustafa S. Applications of flow cytometry on bacteria: Cell cycle kinetics, drug effects, and quantitation of antibody binding. *Cytometry*. 1982; 2: 249–257. <https://doi.org/10.1002/cyto.990020409> PMID: 7035105
19. Fonseca e Silva D, Andrade FF, Gomes R, Silva-Dias A, Martins-Oliveira I, Pérez-Viso B, et al. Ultra-rapid flow cytometry assay for colistin MIC determination in Enterobacteriales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Clin Microbiol Infect*. 2020; 26: 1559.e1-1559.e4. <https://doi.org/10.1016/j.cmi.2020.08.019> PMID: 32835792
20. Ekelund O, Klokhammer Hetland MA, Høyland Löhr I, Schön T, Somajo S. Rapid high-resolution detection of colistin resistance in Gram-negative bacteria using flow cytometry: A comparison with broth microdilution, a commercial screening test and WGS. *J Antimicrob Chemother*. 2021; 76: 3183–3191. <https://doi.org/10.1093/jac/dkab328> PMID: 34477846
21. Mulroney KT, Hall JM, Huang X, Turnbull E, Bzdyl NM, Chakera A, et al. Rapid susceptibility profiling of carbapenem-resistant *Klebsiella pneumoniae*. *Sci Rep*. 2017; 7: 1–13. <https://doi.org/10.1038/s41598-017-0209-3> PMID: 28507322
22. Hatipoglu H, Erman G, Toptan H, Koroglu M, Altindis M. Determination of antibiotic susceptibility of bacteria by flow cytometric method. *World J Microbiol Biotechnol*. 2022; 38: 1–12. <https://doi.org/10.1007/s11274-022-03332-2> PMID: 35781757
23. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, et al. Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc Natl Acad Sci*. 2014; 111. <https://doi.org/10.1073/pnas.1401876111> PMID: 24803433
24. Li H, Zhou X, Huang Y, Liao B, Cheng L, Ren B. Reactive Oxygen Species in Pathogen Clearance: The Killing Mechanisms, the Adaption Response, and the Side Effects. *Front Microbiol*. 2021; 11. <https://doi.org/10.3389/fmicb.2020.622534> PMID: 33613470
25. Memar MY, Ghotoslou R, Samiei M, Adibkia K. Antimicrobial use of reactive oxygen therapy: Current insights. *Infect Drug Resist*. 2018; 11: 567–576. <https://doi.org/10.2147/IDR.S142397> PMID: 29731645
26. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell*. 2007; 130: 797–810. <https://doi.org/10.1016/j.cell.2007.06.049> PMID: 17803904
27. Léger L, Budin-Verneuil A, Cacaci M, Benachour A, Hartke A, Verneuil N. β -Lactam Exposure Triggers Reactive Oxygen Species Formation in *Enterococcus faecalis* via the Respiratory Chain Component DMK. *Cell Rep*. 2019; 29: 2184–2191.e3. <https://doi.org/10.1016/j.celrep.2019.10.080> PMID: 31747593
28. Yeow J, Kaur A, Anscomb MD, New EJ. A novel flavin derivative reveals the impact of glucose on oxidative stress in adipocytes. *Chem Commun*. 2014; 50: 8181–8184. <https://doi.org/10.1039/c4cc03464c> PMID: 24926565
29. Hsueh YH, Tsai PH, Lin KS. pH-dependent antimicrobial properties of copper oxide nanoparticles in *Staphylococcus aureus*. *Int J Mol Sci*. 2017; 18. <https://doi.org/10.3390/ijms18040793> PMID: 28397766
30. Mohiuddin SG, Hoang T, Saba A, Karki P, Orman MA. Identifying Metabolic Inhibitors to Reduce Bacterial Persistence. *Front Microbiol*. 2020; 11: 1–11. <https://doi.org/10.3389/fmicb.2020.00472> PMID: 32292393
31. Orman MA, Henry TC, DeCoste CJ, Brynildsen MP. Analyzing Persister Physiology with Fluorescence-Activated Cell Sorting. 1st ed. In: Michiels J, Fauvert M, editors. *Methods Mol Biol - Bacterial Persistence*. 1st ed. NY: Humana New York; 2016. pp. 83–100. https://doi.org/10.1007/978-1-4939-2854-5_8 PMID: 26468102
32. Murphy MP, Bayir H, Belousov V, Chang CJ, Davies KJA, Davies MJ, et al. Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. *Nat Metab*. 2022; 4: 651–662. <https://doi.org/10.1038/s42255-022-00591-z> PMID: 35760871
33. Belenky P, Ye JD, Porter CBM, Cohen NR, Lobritz MA, Ferrante T, et al. Bactericidal Antibiotics Induce Toxic Metabolic Perturbations that Lead to Cellular Damage. *Cell Rep*. 2015; 13: 968–980. <https://doi.org/10.1016/j.celrep.2015.09.059> PMID: 26565910
34. Ezraty B, Vergnes A, Banzhaf M, Duverger Y, Huguenot A, Brochado AR, et al. Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. *Science (80-)*. 2013; 340: 1583–1587. <https://doi.org/10.1126/science.1238328> PMID: 23812717
35. Jean SS, Lee YL, Liu PY, Lu MC, Ko WC, Hsueh PR. Multicenter surveillance of antimicrobial susceptibilities and resistance mechanisms among Enterobacteriales species and non-fermenting Gram-negative bacteria from different infection sources in Taiwan from 2016 to 2018. *J Microbiol Immunol Infect*. 2022; 55: 463–473. <https://doi.org/10.1016/j.jmii.2021.07.015> PMID: 34503920

36. Benkova M, Soukup O, Marek J. Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *J Appl Microbiol*. 2020; 129: 806–822. <https://doi.org/10.1111/jam.14704> PMID: 32418295
37. Poulikakos P, Tansarli GS, Falagas ME. Combination antibiotic treatment versus monotherapy for multidrug-resistant, extensively drug-resistant, and pandrug-resistant *Acinetobacter* infections: a systematic review. *Eur J Clin Microbiol Infect Dis*. 2014; 33: 1675–1685. <https://doi.org/10.1007/s10096-014-2124-9> PMID: 24832022
38. Sheu CC, Chang YT, Lin SY, Chen YH, Hsueh PR. Infections caused by carbapenem-resistant Enterobacteriaceae: An update on therapeutic options. *Front Microbiol*. 2019;10. <https://doi.org/10.3389/fmicb.2019.00080> PMID: 30761114
39. Tångdén T. Combination antibiotic therapy for multidrug-resistant Gram-negative bacteria. *Ups J Med Sci*. 2014; 119: 149–153. <https://doi.org/10.3109/03009734.2014.899279> PMID: 24666223
40. Verschoor CP, Lelic A, Bramson JL, Bowdish DME. An introduction to automated flow cytometry gating tools and their implementation. *Front Immunol*. 2015; 6: 1–9. <https://doi.org/10.3389/fimmu.2015.00380> PMID: 26284066
41. Sampson TR, Liu X, Schroeder MR, Kraft CS, Burd EM, Weiss DS. Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob Agents Chemother*. 2012; 56: 5642–5649. <https://doi.org/10.1128/AAC.00756-12> PMID: 22908157
42. Dubey V, Gupta R, Pathania R. Targeting Superoxide Dismutase Confers Enhanced Reactive Oxygen Species-Mediated Eradication of Polymyxin B-Induced *Acinetobacter baumannii* Persisters. *Antimicrob Agents Chemother*. 2021; 65: 1–17. <https://doi.org/10.1128/AAC.02180-20> PMID: 33593839
43. Ajiboye TO, Skiebe E, Wilharm G. Contributions of RecA and RecBCD DNA repair pathways to the oxidative stress response and sensitivity of *Acinetobacter baumannii* to antibiotics. *Int J Antimicrob Agents*. 2018; 52: 629–636. <https://doi.org/10.1016/j.ijantimicag.2018.07.022> PMID: 30081139
44. Ajiboye TO, Skiebe E, Wilharm G. Contributions of RecA and RecBCD DNA repair pathways to the oxidative stress response and sensitivity of *Acinetobacter baumannii* to antibiotics. *Int J Antimicrob Agents*. 2018; 52: 629–636. <https://doi.org/10.1016/j.ijantimicag.2018.07.022> PMID: 30081139
45. Ibitoye OB, Ajiboye TO. Ferulic acid potentiates the antibacterial activity of quinolone-based antibiotics against *Acinetobacter baumannii*. *Microb Pathog*. 2019; 126: 393–398. <https://doi.org/10.1016/j.micpath.2018.11.033> PMID: 30476577
46. Ibitoye OB, Ajiboye TO. (+)-Catechin potentiates the oxidative response of *Acinetobacter baumannii* to quinolone-based antibiotics. *Microb Pathog*. 2019; 127: 239–245. <https://doi.org/10.1016/j.micpath.2018.12.012> PMID: 30540925
47. Ajiboye TO, Skiebe E, Wilharm G. Phenolic acids potentiate colistin-mediated killing of *Acinetobacter baumannii* by inducing redox imbalance. *Biomed Pharmacother*. 2018; 101: 737–744. <https://doi.org/10.1016/j.bioph.2018.02.051> PMID: 29524882
48. Deng W, Fu T, Zhang Z, Jiang X, Xie J, Sun H, et al. L-lysine potentiates aminoglycosides against *Acinetobacter baumannii* via regulation of proton motive force and antibiotics uptake. *Emerg Microbes Infect*. 2020; 9: 639–650. <https://doi.org/10.1080/22221751.2020.1740611> PMID: 32192413
49. Lobritz MA, Belenky P, Porter CBM, Gutierrez A, Yang JH, Schwarz EG, et al. Antibiotic efficacy is linked to bacterial cellular respiration. *Proc Natl Acad Sci U S A*. 2015; 112: 8173–8180. <https://doi.org/10.1073/pnas.1509743112> PMID: 26100898
50. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species. *Science* (80-). 2013; 339: 1213–1216. <https://doi.org/10.1126/science.1232688> PMID: 23471410
51. Liu Y, Imlay JA. Cell Death from Antibiotics Without the Involvement of Reactive Oxygen Species. *Science* (80-). 2013; 339: 1210–1213. <https://doi.org/10.1126/science.1232751> PMID: 23471409
52. Fernández-Cuenca F, Gómez-Sánchez M, Rodríguez-Baño J, Martínez-Martínez L, Vila J, Bou G, et al. Epidemiological and clinical features associated with colonisation/infection by *Acinetobacter baumannii* with phenotypic heterogeneous resistance to carbapenems. *Int J Antimicrob Agents*. 2012; 40: 235–238. <https://doi.org/10.1016/j.ijantimicag.2012.05.005> PMID: 22784855
53. Andersson DI, Nicoloff H, Hjort K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat Rev Microbiol*. 2019; 17: 479–496. <https://doi.org/10.1038/s41579-019-0218-1> PMID: 31235888
54. Lim T-P, Teo JQ-M, Goh AW-L, Tan S-X, Koh T-H, Lee WH-L, et al. In Vitro Pharmacodynamics of Fosfomycin against Carbapenem-Resistant *Enterobacter cloacae* and *Klebsiella aerogenes*. *Antimicrob Agents Chemother*. 2020; 64: 1–9. <https://doi.org/10.1128/AAC.00536-20> PMID: 32571821
55. Abe R, Akeda Y, Iida T, Hamada S. Population analysis profiling: is it still the gold standard for the determination of heteroresistance in carbapenemase-producing Enterobacteriaceae? *Int J Antimicrob Agents*. 2022; 60: 10–12. <https://doi.org/10.1016/j.ijantimicag.2022.106644> PMID: 35907596

56. Zhang S, Sun X, Chang W, Dai Y, Ma X. Systematic review and meta-analysis of the epidemiology of vancomycin-intermediate and heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates. *PLoS One*. 2015; 10: 1–22. <https://doi.org/10.1371/journal.pone.0136082> PMID: 26287490
57. El-Halfawy OM, Valvano MA. Antimicrobial heteroresistance: An emerging field in need of clarity. *Clin Microbiol Rev*. 2015; 28: 191–207. <https://doi.org/10.1128/CMR.00058-14> PMID: 25567227