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Characterising the physiological responses of extensively drug resistant (XDR) Acinetobacter baumannii (AB) upon exposure to different antibiotics singly and in combination using flow cytometry (FCM)

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Background: Culture-based methods such as the *in vitro* time-kill studies (TKS) have been widely used to elucidate effective antibiotic combinations against XDR organisms. Such methods, however, cannot detect non-dividing bacterial cells, and may fail to detect heterogeneity (e.g. small populations of resistant bacteria present amongst a majority of susceptible ones). FCM provides rapid quantitative and qualitative analysis of single particles, and has been shown to be useful in determining the physiological responses of bacteria at the single cell level. We employed FCM to assess the activity of multiple antibiotic combinations against XDR-AB, and characterised the physiological responses of XDR-AB upon antibiotic exposure.

Material/methods: Two clinical XDR-AB strains – AB112 [polymyxin B (PB) MIC:1mg/L] and AB8879 (PB MIC:2mg/L)– were employed. TKS were conducted with 5$\log_{10}$ CFU/mL at baseline with clinical achievable concentrations of amikacin (A) (65mg/L), levofloxacin (L) (8mg/L), meropenem (M) (20mg/L), tigecycline (T) (2mg/L), PB (2mg/L) and rifampicin (R) (4mg/L) singly and in two-antibiotic combinations. Prior to TKS, the bacteria were stained with the cell division marker carboxyfluorescein succinimidyl ester (CFSE) (150μM). At each timepoint (0.5, 1, 1.5, 2, 4, 8 and 24h), the following samples were obtained in duplicate: (1) 1mL for viable plating; (2) 1mL for FCM analysis. Propidium iodide (PI) (5mg/L) was used to differentiate between live and dead cells; standardized microspheres were added to facilitate cell counting in FCM. FCM measurements were performed at 488nm.

Results: Bacterial counts obtained using viable plating method and FCM method were similar for all time points when no antibiotics were added (Figure 1A). None of the single antibiotics were bactericidal (defined as ≥3$\log_{10}$ CFU/mL reduction from baseline) against both isolates at 24h upon

viable plating, except for PB against AB112. Against AB8879, M alone exhibited an inhibitory effect on viable plating up to 2h. This corresponded to an observed reduction in bacterial division rate in the first 2h in the FCM analysis (Figure 1B). PB+M and PB+R were bactericidal against AB112 upon viable plating at 24h. Against AB8879, PB+M resulted in an initial bactericidal killing upon viable plating up to 4h, but was followed by regrowth at 24h. FCM analysis of the physiological responses of AB8879 when exposed to PB+M revealed the presence of a viable but non-dividing dormant subpopulation (approximately 4$\log_{10}$ CFU/mL) which persisted in the first 2h; at 24h, bacterial division was observed under FCM, which corresponded to the presence of regrowth observed on viable plating (Figure 1C).

Conclusions: Using FCM, we found that viable plating techniques such as the TKS overestimated bactericidal killing of antibiotic combinations against XDR-AB, due to the development of dormant, non-dividing but viable persisters. The virulence of persisters and their role in the development of pan-drug resistance should be further studied through cell sorting.

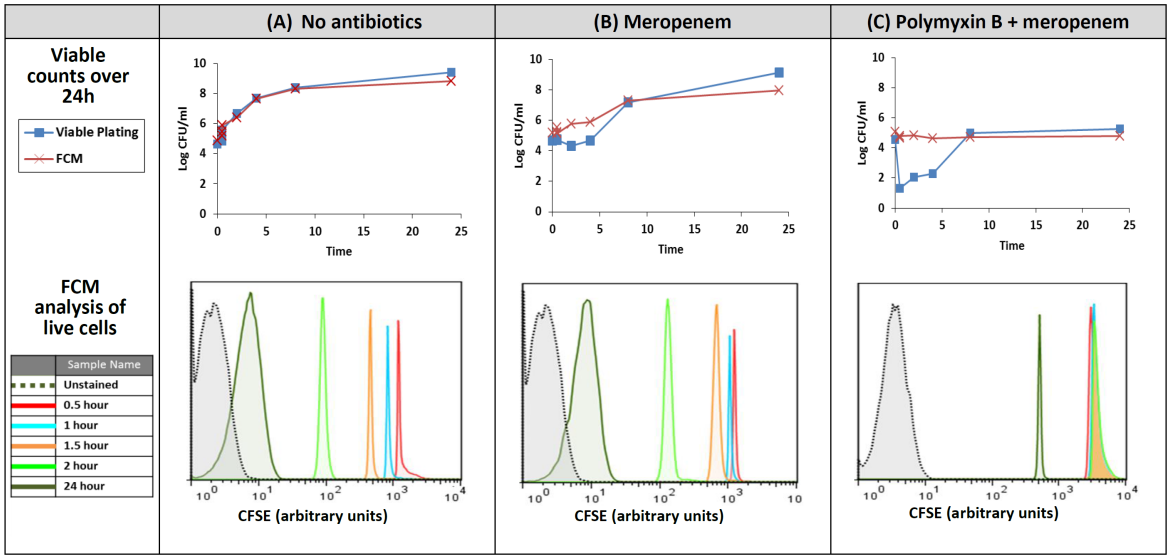

Figure 1: Bacterial counts in viable plating and FCM analysis of AB8879 over 24h TKS and single parameter CFSE histogram analysis of CFSE-stained cells. (A) Normal cell division occurs, resulting in the left shifting of the CFSE peaks with each successive division. (B) Reduction in bacterial division rate, resulting in a smaller magnitude in left shift of the CFSE peaks. (C) Development of a dormant, non-dividing subpopulation (no peak shift).