CASE STUDY

Bacterial phenotyping: Re-tooling antimicrobial research for the 21st century

Introduction

Antimicrobial resistance (AMR) is one of today's major global public health challenges. The number of drug resistant pathogens is increasing worldwide at an alarming rate, placing a heavy burden on healthcare systems.[1,2] Yet despite this, no new classes of antimicrobials have been introduced in the last 30 years.[3]

Recently, there has been renewed investment in the discovery of novel antimicrobials to urgently address the growing number of drug-resistant infections.[4]

Professor Gordon Dougan leads the antimicrobial resistance research programme at the Cambridge Biomedical Research Centre. This is one of twenty UK centres funded by the National Institute for Health Research, which brings together clinical and academic expertise to conduct translational research, transforming scientific breakthroughs into new treatments for patients. The work of his group, which has close links with Addenbrooke's Hospital and the Wellcome Sanger Institute, is multi-faceted, encompassing genomics, diagnostics, bacterial phenotyping, infection models and host-pathogen interactions.

This case study describes how Professor Dougan's team is using the detailed analysis of bacterial phenotypes to investigate adaptive mechanisms of antimicrobial resistance, and screening for novel alternatives to existing antimicrobials.

Our Challenge

"We needed something above and beyond the classical microbiology that has been used for many years to try to identify resistant organisms that appear to be sensitive by classical means.

One of the biggest challenges previously hindering our research was the inability to identify and image a single bacterium within a complex population, at scale."

– Professor Gordon Dougan

The challenge

Current methodologies for analysing compound-induced changes in bacteria can lead to the identification of putative antimicrobial candidates. For example, growth inhibition tests to determine the minimal inhibitory concentration (MIC) of an antimicrobial, though time consuming, have been used for many years, while the more recent introduction of DNA and RNA sequencing has facilitated the analysis of bacterial population structure at a whole-genome level, and enabled genomics and transcriptomics to be linked with MIC data. However, classical methods such as these are ineffective at identifying novel compounds to treat resistant isolates.

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What is a phenotype?

A phenotype is the collected observable traits of an organism. At the level of a cell, this includes properties such as size, shape or molecular content.

Professor Dougan's researchers have been very successful at identifying genes in bacteria, but had limited ability to test the impact of these genes on the behaviour of individual bacterial isolates, e.g. in terms of causing infection or resisting treatment. DNA sequencing could be done at scale but studying the impact on the bacterial phenotype, in particular the ability to image and identify a single bacterium in a complex population, was not scalable. This meant that using classical methods of screening, resistant isolates, which have adapted to persist in hostile environments without alterations to their genome, could be missed within a largely sensitive bacterial population.

Achieving the vision

Professor Dougan and his team are now at the forefront of developing a new method to investigate resistance mechanisms, which uses high-content screening and image analysis as a complement to genomic approaches (Figure 1) to discover new ways of identifying and treating bacteria which are resistant to normal treatments using antimicrobials or antibodies.

This approach has transformed the scale and sensitivity by which bacterial populations can be analysed and importantly, enables the study of correlations between genotypic and phenotypic changes. HCS can validate the RNAseq data and reveal hits that might not seem relevant when looking only at the sequencing data but might be important in identifying new targets.

High-content imaging is performed using the Opera Phenix™ high-content screening system (Revvity). This state-of-the-art system was selected as it offers the high-quality image resolution required to visualize single bacteria within a population, together with analysis software that provides unique information on the shape and specific properties of each cell.

Professor Dougan believes that the Opera Phenix system will be valuable in both characterizing the effect of antimicrobials, as well as in discovering new antimicrobials and antibodies that can kill bacteria.

What is high-content screening and why use it?

High-content screening (HCS), which combines automated fluorescence microscopy with quantitative image analysis, allows the acquisition of unbiased multi-parametric data at the single cell level.[5]

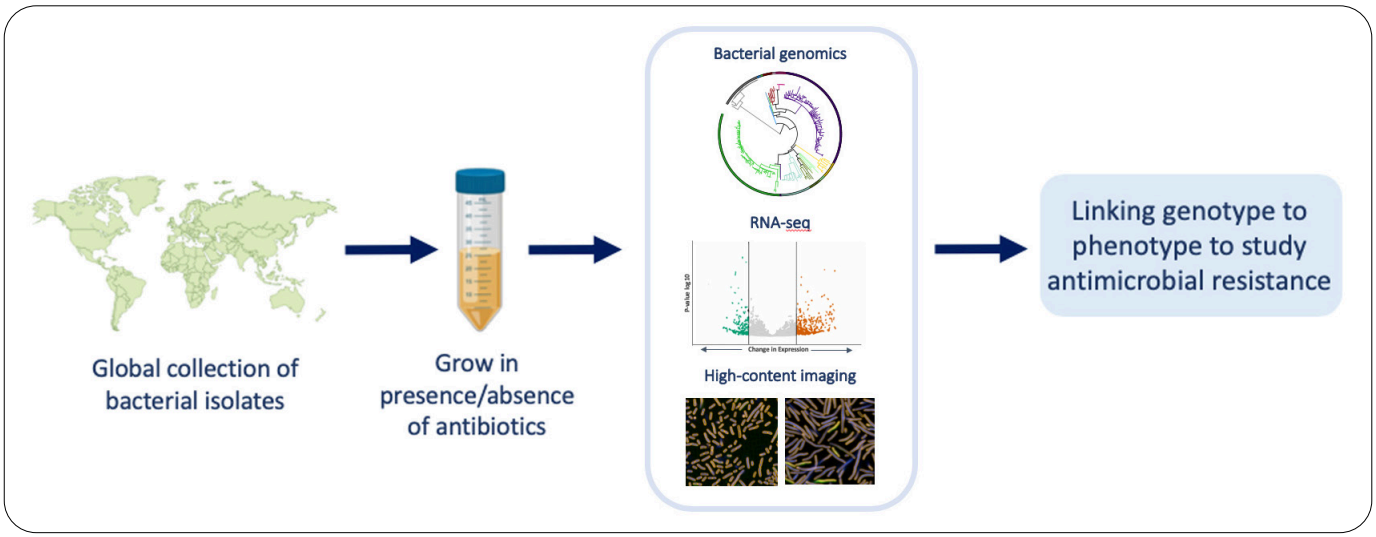

Figure 1: Linking genotype to phenotype to study antimicrobial resistance. Genomic, RNAseq, and high content data can be correlated to give a more complete picture of how antimicrobials work against bacteria. This can potentially lead to a better understanding of how bacteria develop resistance and reveal novel targets for new antibacterial compounds. Bacterial genomics tracks transmission and acquisition of genes that determine antimicrobial resistance over time. RNA-sequencing/transcriptomics provides insight into how bacteria react, or protect themselves from antimicrobials, as well as the antimicrobial mechanism of action. High-content imaging/screening detects and quantifies how phenotype changes as bacteria resist or persist in the presence of antimicrobial. The schematic was created with icons from BioRender.com

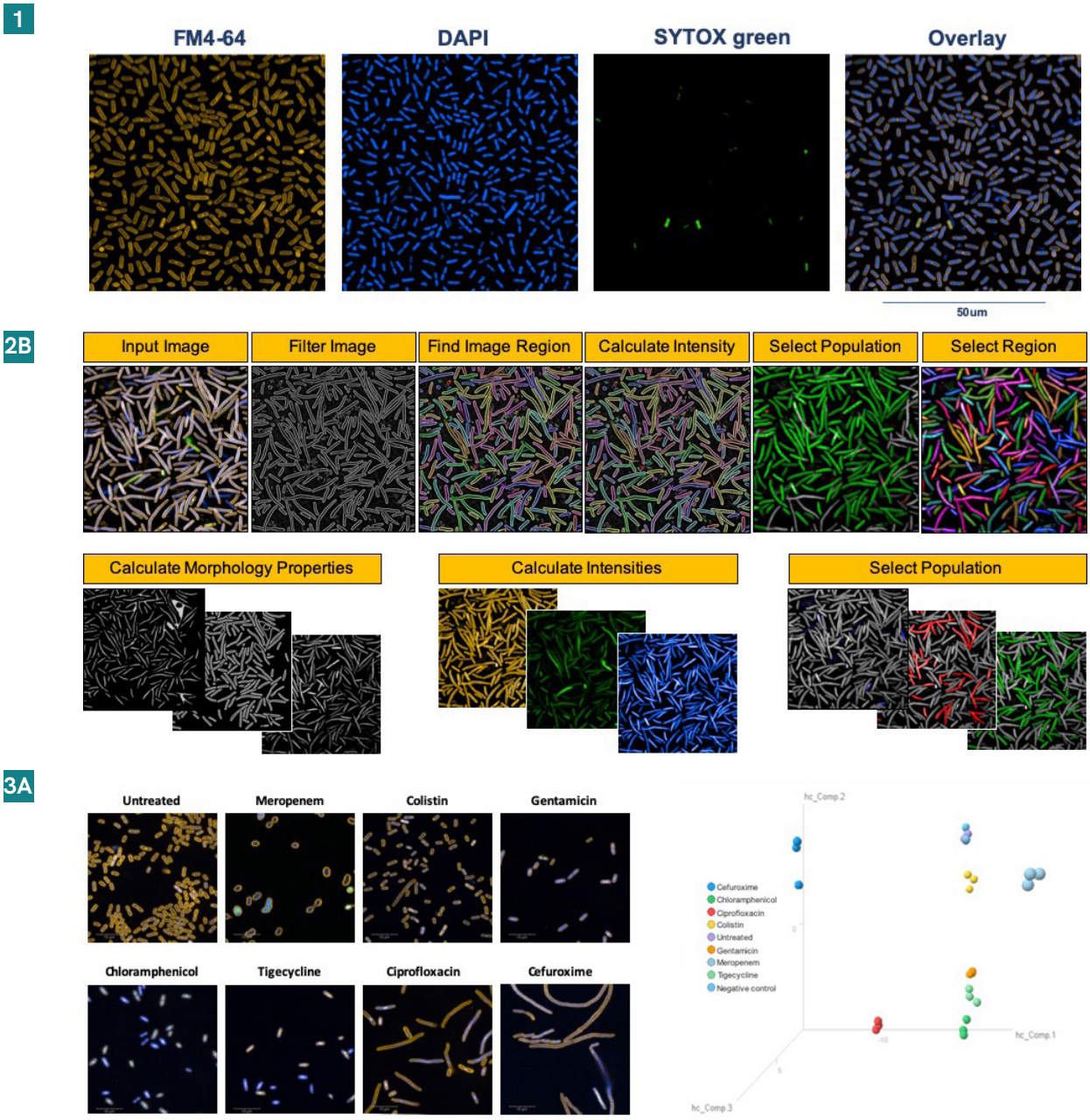

Figure 2. Phenotypic screening workflow, this example focuses on Klebsiella pneumoniae treated with different classes of antimicrobials at x5 MIC but the method can be transferred to other bacterial strains.

1. Imaging. Bacteria were stained by DAPI (nucleic acid, live), SYTOX Green (nucleic acid, dead) and FM4-64 (membrane) in PhenoPlate™ 96-well microplates (formerly known as CellCarrier Ultra) and imaged on the Opera Phenix™ using the 63x water immersion objective (10 fields and 3 Z-planes per well). Automated acquisition of a 96-well plate took 1 hour.

2. Image Analysis. A ready made building block sequence within Harmony enable bacteria to be identified within each image (2A). Once segmented each bacterium is fully characterized through calculating basic and advanced morphology properties as well as textural and intensity (2B). Finally, the built-in machine learning tools define single bacterial cells and exclude any artifacts.

3. Data Visualization. Images show how bacterial phenotype differs when treated with different classes of antimicrobial (3A). Principal component analysis (PCA) was generated using High-Content Profiler in Tibco Spotfire. PCA is a visualization method especially suited for multiparametric data sets such as phenotypic profiles. It reduces the complexity of the multi-parametric data sets showing clearly the similarities and differences between bacterial responses to drugs. This type of approach could help find antimicrobials with novel mechanisms of action through comparison with known classes of antimicrobials (3B) Data courtesy of Dr. Ben Warne.

The impact on our research

With high content screening, we can look at mechanisms of kill. For example, for an antibiotic, we can look at a population of bacteria and watch the killing process as it actually happens, and look for bacteria that escape kill. This has allowed us to design experiments where we can look for mechanisms of how a bacterium is escaping, look at genetic map of the bacterium then maybe make a mutation in that bacterium and re-test if we suspect a particular gene is influencing resistance"

– Professor Gordon Dougan

Since introducing the Opera Phenix system, Professor Dougan's team has optimized its workflow (Figure 2) by developing a series of standard operating practices when preparing different bacteria for imaging analysis. This ensures reproducibility in their experiments and generates robust, validated data. The Opera Phenix system captures these data in a standardized way and enables comparison between individual bacteria, thereby limiting the amount of experimental error in the system.

The impact

Using high-content imaging, researchers in Professor Dougan's laboratory can observe the killing process in a bacterial population in real time, following exposure to an antimicrobial compound, enabling them to identify resistant bacteria. This allows experiments to be designed to then explore the mechanisms by which resistance occurs and identify the pathway through which this has evolved, using phylogenetic analysis.

Professor Dougan envisages that the Opera Phenix high-content screening system will ultimately transform the way microbes are phenotyped, enabling his team to continue increasing the efficiency achieved with this high-throughput approach by introducing other types of support, such as robotised systems, or machine learning and artificial intelligence to further improve and accelerate phenotyping.

Funding bodies have been quick to see the potential value of this methodology in developing new therapeutics, and there is also considerable interest from pharmaceutical and biotechnology companies.

Looking to the future

There is rapidly growing interest in the role of the microbiome in human health and disease.[6] Disruptions to the microbiome are thought to play a key role in a number of conditions including irritable bowel disease, metabolic diseases, cancer and Parkinson's disease. The use of high-content screening and imaging analysis would enable more rapid phenotyping of the microbiota that comprise the microbiome. This could pave the way to personalization of therapy or the creation of an artificial microbiome, which may offer an alternative to faecal microbiota transplants in the treatment of gastrointestinal infections and other conditions.

In addition, microbial profiling using high-throughput platforms could have a significant impact in the clinic in the longer-term future. The time to identify an infection could be shortened considerably, thereby reducing the need to decide on antimicrobial treatment empirically, while also supporting selection of the most appropriate antimicrobial, potentially reducing reliance on broad-spectrum agents.

Conclusion

High-content analysis is well-established in the study of eukaryotic cells. Here, Prof. Dougan and his team have demonstrated how this technology can also be applied to prokaryotic cells, and how advances in image quality and image analysis have enabled detailed phenotypic profiling of individual bacteria at scale. High-content analysis:

- Allows single resistant bacteria to be identified within populations, unlike genomic approaches.
- Provides new perspectives in the search for new targets.
- Can give insights into the mechanism of action of novel compounds.

Acknowledgements

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