Celebrating 25 years of advances in microbiology

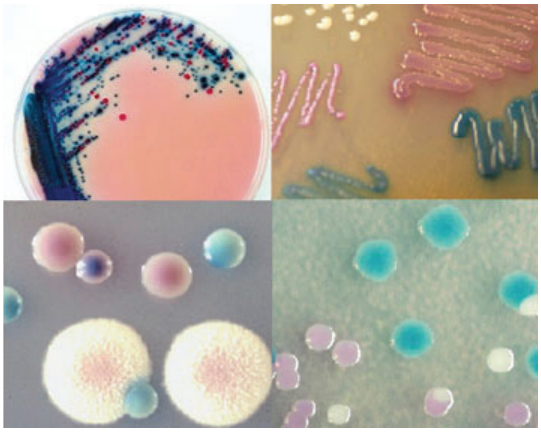
Julie A Creighton

There have been numerous amazing advances in microbiology over the last 25 years, many of which have been improvements on existing technology, but some have been innovative ideas stemming from scientists' naturally inquisitive minds. I have chosen four areas to highlight, which I think have had the greatest impact on the routine clinical microbiology laboratory: chromogenic agar, mass spectrometry, antimicrobial resistance, and whole genome sequencing.

Chromogenic agar

Routine sample processing in microbiology still relies on growing bacteria on solid media. The introduction of chromogenic agar has had a great impact on diagnostic clinical microbiology over the last 25 years. Various types of chromogenic agar are used across most areas of sample processing for the identification of diverse species.

In 1989 Dr Alain Rambach, a French scientist, patented a chromogenic media, known as Rambach agar, for the specific isolation of *Salmonella* species from faecal specimens. The media was a success, enabling the CHROMagar™ product line to expand and generate a plethora of industry competitors. The basic principle of chromogenic agar relies on targeting the presence of specific enzymes in bacteria e.g. beta-glucuronidase is specific to *E. coli* and active in approximately 95% of strains. The solid chromogenic media contains soluble molecules, called chromogens, which are colourless. The chromogens are composed of a substrate coupled with a chromophore (e.g. pH indicator). If a specific enzyme is present in the organism of interest, it reacts with the substrate, releasing the chromophore, enabling the unconjugated chromophore to exhibit its distinctive colour. The chromophore must be relatively insoluble so that it forms a coloured precipitate immediately around the targeted organism.



Chromogenic agar has three main areas of application (i) detection of enteric pathogens from faecal samples, e.g. *Salmonella* spp., *Shigella* spp., and Shiga Toxin-Producing *E. coli*, (ii) detection of specific pathogens from specific sample types, e.g. *Candida* spp. or Group B Streptococcus from vaginal swabs, or *S. aureus* from sputum samples, (iii) detection of antimicrobial resistant bacteria from screening samples, e.g. MRSA, VRE, ESBL or CPE. These applications allow the laboratory to quickly and efficiently "screen" for a specific organism or a group of bacteria (although confirmation of identification is still required), saving time and resources. The benefit of rapid pathogen identification enables timely follow up of healthcare interventions such as antibiotic treatment and implementation of infection control procedures or public health measures.

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry)

Bacterial and fungal identification has traditionally been based on phenotypic tests such as biochemical reactions, culture characteristics, and Gram stain pattern. Some identification tests can be rapid, for example the Spot Indole test to detect the enzyme tryptophanase in *E. coli*; however, many other tests take hours, overnight, or even days if the bacteria or fungi is fastidious or slow growing.

Mass spectrometry has been used for decades but its application to bacterial identification was limited due to low volatility and thermal instability of large molecules such as proteins and carbohydrates. The matrix-assisted laser desorption/ionization (MALDI) technique utilises a chemical matrix material which can absorb energy from the laser light, resulting in the creation of ions from large molecules without degradation. The laser is shot multiple times, which improves the signal-to-noise ratio, thus increasing the accuracy of the molar mass determination. The time-of-flight (TOF) analyser measures the traverse of particles from time of ionisation to the recording detector. The overall mass spectrum is analysed according to molecule mass charge and travel time. The resulting profile is compared to stored database reference profiles, generating a bacterial or fungal identification to either genera or species level. A numeric score helps to assess how closely the organism identification matches the reference spectra. A low score might indicate that the organism is not in the database, or that the biomass is insufficient (due to low density of organisms or immature growth).

MALDI-TOF MS technology has revolutionised organism identification in clinical microbiology over the last decade. Medical laboratories in New Zealand now have, or have access to, a MALDI-TOF instrument; either the Bruker-MS or the bioMérieux Vitek-MS. The instruments have high throughput, are accurate, low cost, and reduce waste, with identification of bacteria and yeast achieved in minutes. The identification of filamentous fungi can be more complex due to variable protein phenotypes. However, improved databases and the ability to have a user-developed database has improved accuracy and performance for filamentous fungi. The routine MALDI procedure is simple, starting with applying a small amount of organism directly onto an edged circle on a metal target plate. The smeared preparation is then overlaid with a 70% formic acid solution for initial extraction (step optional), air dried, then overlaid with matrix solution. The plate is dried again before placing into the MALDI-TOF instrument and running the chosen application. Different preparation protocols can be used for fluids such as blood cultures or urine, or additional extraction steps for fastidious organisms, or to obtain a better identification score. If the instrument is interfaced, then the organism identification result can be sent directly to the laboratory information system.

MALDI-TOF spectra can also be used for other applications, such as detection of a specific biomarker (e.g. to predict methicillin resistance in *S. aureus*), or for the detection of antibiotic degradation (e.g. detection of the carbapenem hydrolysis product following degradation by a carbapenemase enzyme in carbapenem resistant Enterobacterales), or for organism subtyping. There is no doubt that MALDI-TOF is an indispensable tool for microbiology and its future use will only be enhanced with continued library database extensions and new applications.

Antimicrobial resistance (AMR)

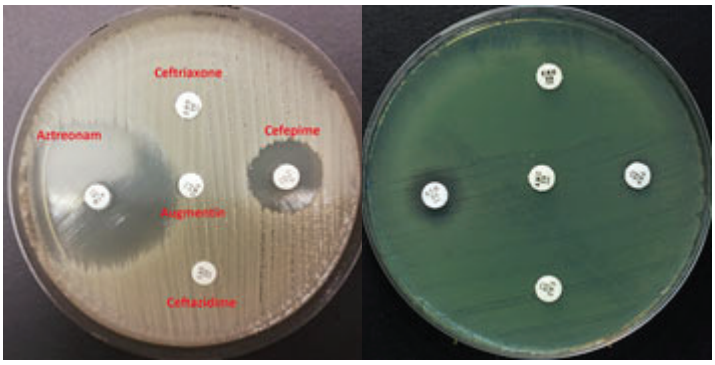
The escalating rates of antimicrobial resistance over the last couple of decades is a global health threat, affecting humans, animals, the environment, and economic productivity. Infections caused by multi-resistant organisms are associated with high morbidity and mortality rates. Drug resistance can be found in many microbes, including *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, HIV, malarial parasites, and fungi. However, it is the rapid emergence of resistance and the expanding variety of resistance determinants in Gram-negative bacteria, particularly the Enterobacterales, that is classified as an urgent threat by the WHO. Enterobacterales such as *E. coli* and *Klebsiella pneumoniae* are common gut colonisers and frequently cause community-acquired infections such as urinary tract infections, blood stream infections, gastroenteritis, and hospital-acquired infections.

Extended-spectrum β-lactamases (ESBLs) are enzymes that can hydrolyse most β-lactam antibiotics except for carbapenems. Interspecies dissemination of ESBLs is facilitated by mobile genetic elements such as integrons, transposons, and plasmids. Plasmids can have a broad host range and frequently harbour resistance determinants to other antibiotic classes, leaving few treatment options. ESBLs were first described in 1986, but in New Zealand they were infrequently found, even up until 2000 (only 27 isolates reported to ESR). Since then, the prevalence of ESBL-producing Enterobacterales has rapidly accelerated. The most recent ESR survey was completed in 2016, collecting 521 ESBL isolates during a one-month period, from clinical samples only, suggesting a prevalence rate of 11.1/100,000 population. These numbers will be even more alarming today. Worldwide studies have shown the dissemination of genes encoding ESBLs in animals (farm, domestic, wild) and in the environment, creating community reservoirs for continued dispersion.

A consequence of high rates of ESBL-producing pathogens has been a corresponding increase in the use of carbapenem antibiotics, which in turn has led to the rapid selection of carbapenem resistant organisms. Carbapenem resistance can be due to a variety of resistance mechanisms, the most concerning of which are carbapenemase enzymes - found in various bacterial species, carried on a broad range of plasmid hosts, and are widely disseminated in nature and healthcare settings. Carbapenemases emerged decades ago but it wasn't until the 2000's that they started to proliferate (KPC along the Eastern seaboard of the United States, NDM in India). The prevalence and diversity of carbapenemases have increased worldwide and they are now endemic in many regions of the globe, presenting an enormous challenge to the health sector.

In New Zealand the first report of a carbapenemase-producing Enterobacterales (CPE) was in 2009. Between the years 2009 to 2014 only 35 CPE were identified, all most likely acquired overseas (hospitalisation or travel associated). Since 2014 the total number of CPE isolated each year has been steadily increasing, along with an expanding range of enzyme types (although still dominated by NDM and OXA-48-like), in a variety of Enterobacterales species. Overseas travel, especially from the Indian subcontinent, is still the highest risk factor for acquisition. Worryingly there have been several episodes of transmission events in NZ healthcare facilities as well as community transmissions. It remains to be seen if NZ's border restrictions have any impact on slowing our rising prevalence rates.

To curb AMR, future priorities should include faster laboratory detection, strengthening infection control bundles and international efforts to stimulate the research and development of innovative antimicrobials.



NDM-producing *Klebsiella oxytoca* VIM-producing *Pseudomonas aeruginosa*

Whole genome sequencing (WGS)

The technology that has rapidly manifested around DNA sequencing is a game changer for microbiology. 25 years ago, pulsed-field gel electrophoresis (PFGE) was at the forefront of the molecular epidemiology era. It was considered the gold standard for assessing genetic relatedness among strains of bacteria following a possible hospital-acquired infection or disease outbreak (e.g MRSA and *Pseudomonas aeruginosa*). However, PFGE is time consuming, labour intensive, and is technically challenging. In addition, it has limited discriminatory power, especially with organisms that undergo significant amounts of horizontal gene transfer.

The human genome project, which identified and mapped the DNA sequence of the human genome, was started in 1990, but it took until 2003 to be completed. Since then rapid advances in the field of whole genome sequencing (WGS) have evolved, including the introduction of next-generation sequencing (NGS), meaning that an entire human genome could be sequenced within a day. WGS could distinguish bacterial relatedness at the nucleotide level, offering far greater resolution than PFGE. However, at the early stages of development instrumentation was very expensive, methods were not standardised and obtaining accurate data analysis could be difficult. Over the last few years the use of WGS has become more common place, especially in large institutions and universities, and we are now at the beginning stages of a transformation into mainstream clinical microbiology. WGS has proved to be a powerful tool for epidemiological investigations of disease outbreaks, with

improving technology allowing real-time analysis, detecting epidemiological risks as they arise. Since the SARS-CoV-2 epidemic, everyone has become familiar with the term “genomic sequencing”!

Aside from epidemiology, NGS can be utilised for resistance profiling. This technology is often superior to challenging phenotypic methods, but it relies on good sequence data and known database – which means that novel resistance mechanisms might be missed. WGS currently is too slow and expensive to replace routine antimicrobial susceptibility testing methods, and it is not suitable for treatment guidelines as gene absence cannot always predict susceptibility.

Metagenomic testing has had intense publicity in recent years, particularly around the gut microbiome. WGS performed on cultured bacteria is a current bottle neck in timely reporting for clinical microbiology. Metagenomic approaches, where the collective genetic material is analysed (e.g. detection of dysbiosis in vaginal discharge, or detection of organisms in sterile fluids) could be an exciting future application, providing rapid non-biased culture-independent diagnosis. Current limitations include method standardisation, issues with false positive results from contaminating DNA and samples with low biomass.

Nanopore, third generation sequencing, is the latest sensation. The MinION, Oxford Nanopore Technologies, uses a protein flow cell and electrical conductivity changes to identify DNA bases, producing long-read sequences. The MinION is small and can plug into a USB port on a laptop computer, making it a highly portable real-time sequencing device. IThe recently released “Flongle” is an adaptor for the MinION, designed for smaller DNA/RNA sequencing tests and lower cost. This technology could potentially be used for targeted sequencing for bacterial resistance genes, metagenomics or organism identification. With simultaneous sample processing, continued software and performance developments and cost decreases, this technology has the potential to transform our routine molecular workflow.

Oxford Nanopore MinION

If the last 25 years is anything to go by, the future for microbiology is looking incredibly exciting!

AUTHOR INFORMATION

Julie A Creighton, DipMLT FNZIMLS, Senior Medical Laboratory Scientist[1] and Clinical Lecturer[2]

[1]Microbiology Laboratory, Canterbury Health Laboratories, Christchurch
[2]University of Otago, Christchurch

Correspondence: Julie Creighton.
Email: julie.creighton@cdhb.health.nz