Introducing NGS to the clinical microbiology laboratory: an update

The Annual Scientific Conference of the British Society for Microbial Technology (BSMT), due to take place last May, was cancelled. In this article, written on behalf of the BSMT, Mark Wilks collaborates with Adela Medina to summarise the presentation she planned to give at this year's event.

One of the most eagerly anticipated presentations to the British Society for Microbial Technology (BSMT) Annual Scientific Conference 2020 was to have been by Adela Medina, describing work using Oxford Nanopore sequencing directly on clinical specimens from patients to identify rapidly any bacteria, fungi or DNA viruses present. Over the past five or so years, there has been increasing mention of DNA sequencing in clinical microbiology laboratories. The introduction of next-generation sequencing (NGS) in clinical microbiology laboratories has increasingly been advocated as a practical approach for the diagnosis of infectious disease as well as tracking outbreaks, typing organisms, and in metagenomic studies.

The ability to rapidly detect non-culturable bacteria and those that take a long time to grow using routine methods in a single test is highly attractive. Improvements in the accuracy of technology and decreasing costs make it an increasingly attractive proposition. Up until now, however, there have been two major obstacles – capital costs remain high, and the need for bioinformatics expertise. These factors have limited the introduction of the technology into the diagnostic clinical microbiology laboratory. Adela Medina's work used the Oxford Nanopore MinION system, which is a USB-sized cell connected to a laptop or desktop computer. There is even a version that connects to a mobile phone. The use of a disposable cell containing hundreds of pores through which the DNA strands migrate avoids the need for the initial high capital expenditure, which has been one of the two major barriers to adoption.

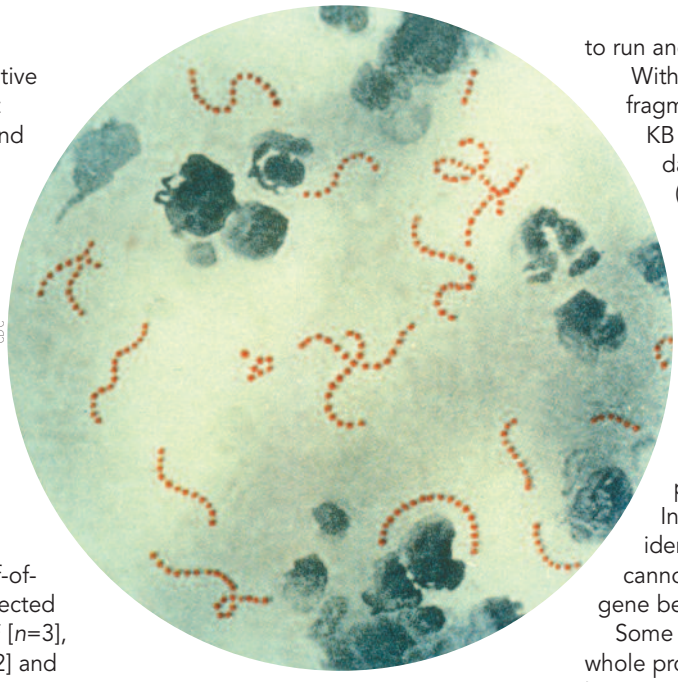
The advantage of NGS compared with other molecular techniques like 16S polymerase chain reaction (PCR) targeted at a specific gene is that it allows the sequencing and identification of numerous pathogens in a single run and it is 'agnostic' (ie there is no need to target specific pathogens for example by primer selection in advance).

On the other hand, lack of the target specificity together with high sensitivity means that NGS is particularly susceptible to contamination, which is a much greater problem in samples with low biomass – sites which are normally described as 'sterile'. The use of control samples sequenced at the same time as the clinical samples in different steps of the process is essential to control contaminations in the laboratory

The Oxford Nanopore MinION system, a USB-sized cell that can be connected to a laptop, desktop computer or even a mobile phone.

environment and minimise cross-contaminations. The normal negative controls used for PCR are just not adequate. Failure to realise this and not use the appropriate controls has led to publication of some literally incredible results, where every site in the human body appears to have a complex microbiota, which in fact merely reflects reagent contamination. Several guidelines on how to avoid this mistake by elaborate use of controls have now been published.

Barts Health NHS Trust processes approximately 550,000 samples per year. In a small proof-of-principle study, Adela Medina selected 10 samples (abscesses [*n*=3], CSF [*n*=3], hip tissue [*n*=1], EDTA blood [*n*=2] and prosthetic valve [*n*=1]) from patients in whom a diagnosis was not obtained by routine laboratory tests or was not concordant with the clinical suspicion, and directly sequenced.



In one of the abscess case, *Streptococcus pyogenes*, seen here as chain-linked organisms, had not been isolated by culture, but detection by the Nanopore MinION system made sense of the clinical situation and allowed antibiotics to be rationalised (Pappenheim stain, original magnification x900).

Depleting human DNA from the specimen before sequencing is key

Sample preparation is relatively simple. It is key to try and deplete human DNA from the specimen. The concentration of human DNA even in a specimen that has a high concentration of bacteria greatly exceeds the concentration of bacterial DNA – often by a factor of a million, or even a billion in the case of blood. All samples (400 µL) are treated to deplete human DNA from the sample with a saponin-based differential lysis/endonuclease digestion method. The DNA from the organism present in the sample is then extracted on a Qiasymphony (Qiagen) or another automated platform available in all large laboratories. In fact, manual extraction is also possible, which makes the approach even more flexible,

At this stage with 'conventional' larger DNA sequencers you have to think about batching samples to make it economically possible to run. This means that you have to sacrifice speed in favour of affordability, and so one of the key advantages of sequencing is lost. With the Nanopore system, it is quite possible to run a couple of samples at a time. In this case, the extracted samples were processed using the Rapid PCR Barcoding Kit (ONT) according to the manufacturer's protocol, and four samples were tested in the same sequencing run, together with a negative control (nuclease-free water) included in the run.

It seems counter-intuitive, but there is no fixed time for the sequencing run. So, if you were running pure bacteria say and you wanted to type them, you would want the most accurate results, and let the sample run for a long time (24-48 hours). In Adela's study, samples were run for only two hours, as this should give enough information to detect any bacteria present

No need for specialised bioinformatic training

The next problem with NGS is how to interpret the data. Conventional DNA sequencers analyse short DNA sequences – perhaps as short as 50–70 bp and obtain an identification by multiple alignments and overlaps to obtain an identification. This process takes time; perhaps up to 48 hours to analyse the massive amount of data obtained. It is not just a matter of time; there is the bigger problem of bioinformatic expertise to run and understand the analysis.

With Oxford Nanopore, much longer fragments of DNA, often up to 2–3 KB or more, are sequenced. The data are analysed using WIMP (what's in my pot), a proprietary piece of software that rapidly identifies the long pieces of DNA sequenced. There is no need for specialised bioinformatic training to run this programme. The identifications are obtained and displayed in real time as the DNA samples are analysed passing through the nanopores. In addition, resistance markers are identified as well, although you cannot say that a particular resistance gene belongs to a particular organism

Some authors have claimed that the whole process can take as little as six hours; in the example reported here, the turnaround time for the whole process was about 10 hours, which is still remarkably short. The times taken for each stage were two hours for human DNA depletion, one hour for microbial DNA extraction, five hours for library preparation and PCR, and two hours for actual sequencing.

What about the actual results? These were to have been presented at the BSMT May meeting (postponed until May 2021) in a lecture entitled *Bringing in NGS to the clinical microbiology laboratory without high capital expenditure or specialised bioinformatics knowledge*, but it is worth looking at a couple of results to get a flavour of them and to show how the technology can help. As a rule, sequencing obtains all the bacteria that are cultured and often a lot more.

The first sample to be analysed was a CSF from a patient with meningitis. The specimen was culture-negative and significantly also negative by 16S PCR, but on sequencing *Streptococcus pneumoniae* was detected. Although meningitis was suspected, the detection of *S. pneumoniae* allowed anti-infective therapy to be rationalised (allowing adjunctive corticosteroids to be prescribed). In addition, identification led to discussion with the Ear, Nose and Throat (ENT) team about where *S. pneumoniae* might have originated. leading to surgery for mastoiditis to achieve source control. Furthermore, immunological investigations were carried out to explore why this patient developed invasive pneumococcal infection.

Sample 2, a breast abscess, was quite different. Here, a large number of bacteria were cultured – *Enterobacter cloacae*, *Acinetobacter* spp., coagulase-

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negative *Staphylococcus* and mixed anaerobes. However, in this case, the clinicians thought the organisms obtained by culture were unlikely to be the causative pathogen, so MinION sequencing was requested. This showed the 'alphabet soup' of bacteria detected by culture but demonstrated the presence of *Streptococcus pyogenes* DNA. *S. pyogenes* had not been detected by culture, but made sense of the clinical situation and allowed antibiotics to be rationalised. Barrier nursing was carried out until the surgical team was satisfied that all necrotic tissue has been debrided and the case was notified as an invasive group A *Streptococcus* infection to the local public health authorities.

Can we afford it?

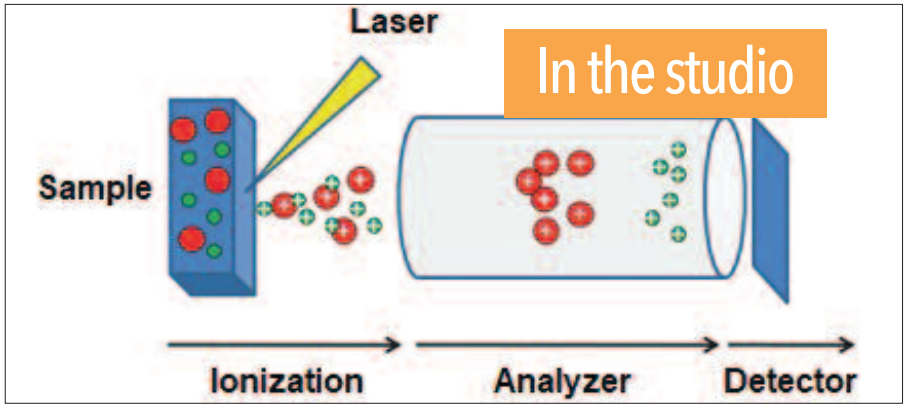
So, how much does all this cost? The cost per sample is around £100 in reagents, and it is likely to become cheaper over time. Labour costs are hugely variable and quite difficult to measure accurately. The skill levels required to set up and implement the technology are high, but, once adopted by a typical large diagnostic laboratory, depend primarily on meticulous technique and adherence to standard operating procedures rather than a high level of molecular biology expertise.

Obviously £100 a test is a lot and would only be considered for a small number of cases, perhaps those negative by all other tests, as in the meningitis case mentioned above. Nevertheless, if £100 a test seems a lot, it is sobering to reflect that one company. Karius in the USA, is offering a direct sequencing service from blood at a cost of $2000 per test! Specimens are sent to a central laboratory for testing, so the whole process takes 24–48 hours, in contrast to the 10 hours for Oxford Nanopore sequencing.

Oxford Nanopore sequencing emerged as a viable technology for laboratories at the same time as the SARS-CoV-2 coronavirus pandemic made the need for rapid identification of bacteria and fungi even more important. One of the main concerns in COVID-19 patients admitted to ICU has been possible secondary infection, mainly with bacteria such as coliforms or *Pseudomonas*; *Aspergillus* is also a potential problem due to immuno-suppression received by these patients to deal with the hyper-inflammatory reaction.

When the pandemic hit the UK, a pilot research project was started to detect secondary infections in an ICU patient with a COVID-19 diagnosis at St Thomas' Hospital. A multidisciplinary team, from the Centre for Clinical Infection and Diagnostics Research (CIDR) and the Viapath clinical scientist team, now including Adela Medina, have been working on this project. Hopefully, delegates will receive a report of this at the next BSMT Scientific Meeting.

Meanwhile, for further reading, an excellent starting point is an article by Dr Justin O' Grady's group from the University of East Anglia describing the use of nanopore technology in the diagnosis of respiratory tract infection. Some readers may remember that Dr O'Grady spoke at the BSMT Annual Scientific Conference in 2018 on the use of Nanopore sequencing.



Mass spectrometry in the form of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry has quickly become accepted into the diagnostic laboratory, completely transforming the way bacteria are identified.

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Beyond MALDI-TOF

Finally, we should mention the fact that it is not only in DNA sequencing that microbiology is continuing to advance at a rapid rate, Mass spectrometry in the form of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has quickly become accepted into the diagnostic laboratory, completely transforming the way bacteria are identified.

It looks as though 'the next big thing' is about to be launched by Thermo Fisher Scientific in the form of a high-resolution MS system, which, unlike existing systems, was designed from the bottom up for clinical microbiology laboratories. The increased resolution should allow the differentiation of even closely related species (eg *Escherishia coli* and *Shigella*) and should appeal to clinical laboratories.

Equally important, it avoids the 'clunkiness' of present methods of identifying bacteria (eg from positive blood cultures), where the need for several manual steps outside the instrument has handicapped take up of the various kits. Designing a whole system from scratch, rather than adapting an existing instrument, takes longer but does mean more time to provide fully automated analysis and full sample traceability. This should minimise laborious manual steps and streamline the whole operation.

Perhaps most importantly, for the first time, simple detection of some key resistance markers within the instrument, without the need for further external steps that can be time-consuming and labour-intensive, is also possible. The ability to rapidly and simply identify an organism from a blood culture as well as detect selected resistance markers are major steps forward. The system has just been CE-marked so we should be hearing a lot more about it in the next few months.

Reference

1 Charalampous T, Kay GL, Richardson H *et al.* Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol.*2019; **37** (7): 783–92. doi: 10.1038/s41587-019-0156-5.

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Further information on the British Society for Microbial Technology may be found online (www.bsmt.org.uk).