Novel diagnostic technologies for clinical and frontline use

Advanced diagnostics based on molecular markers and analysis technologies has been improving diagnosis across a wide range of diseases

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Misdiagnosis is a major problem at all levels of health care causing unnecessary pain or even death as it prevents efficient, timely, and adequate treatment. In the case of infectious diseases, for instance, identification of the wrong pathogen might lead to incorrect prescription of antibiotics. While reliable data on the full extent of misdiagnosis are elusive, some evidence of the problem's scale came from a meta-analysis of deaths within US intensive care units (ICUs), which found that 40,500 adult patients in the USA may die each year as a result of a misdiagnosis [1]. An earlier, more comprehensive, study based on searching the MEDLINE (1950–2007) and EMBASE (1980–2007) electronic databases for articles about diagnostic error or delay in primary care also found that misdiagnosis was a significant issue, although the reasons varied with each condition [2].

Moreover, many diseases, in particular rare diseases, are hard to diagnose as physicians are often unfamiliar with the symptoms; alternatively, the symptoms might be vague or untypical, as can happen with some autoimmune disorders. Many cancers in their early stages do not even show any symptoms, which prevents timely and effective treatment. There are also complex disorders that present highly varied symptoms that can lead doctors in the wrong direction, at least at first.

Not surprisingly, there is an immense interest in molecular-level diagnostics that promise to deliver far more accurate and rapid diagnoses without relying on interpreting symptoms. While this can raise new challenges, such as poor-quality samples or

analyzing complex data sets, molecular diagnostics has made progress across a range of clinical conditions, including infectious diseases and some cancers, through identification of characteristic molecular markers. A wide variety of diseases could also be diagnosed using next-generation sequencing (NGS), including solid cancers or rare diseases of genetic origin. NGS in combination with metagenomics can also be applied to more complex samples—for instance from the gut—to analyze whole microbiota and diagnose a wide range of conditions, such as intestinal disorders or skin problems.

Frontline diagnostics

While some applications involve complex analysis and expensive equipment, progress is also being made toward smaller systems, especially for basic pathogen diagnosis. “In the case of infection, you want to know first whether a patient has a bacterium rather than a virus and if so find out whether it has resistance to antibiotics”, commented Jim Huggett, who works in analytical microbiology at the University of Surrey, UK. More informative and advanced tests can then be applied higher up the healthcare systems, according to Rosanna Peeling, Chair of diagnostics research at the London School of Hygiene and Tropical Medicine. “WHO (World Health Organization) now has a pyramid of tests for different levels”, she said. The idea is to provide the best available treatment immediately for acute cases or make a referral to a specialist clinic. This is particularly relevant for tuberculosis to confirm the initial diagnosis through

identification of the specific strain of the causative mycobacterium.

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Oxford Nanopore, a private company headquartered near Oxford, UK, has been pioneering nanoscale technologies for simple and rapid DNA analysis. Its first product called MinION, which uses pore-forming proteins in membranes on a dongle that plugs into the USB port of a laptop for power and communication, has been commercially available since May 2015. It pulls a DNA molecule through a nanopore and measures changes in the electric potential across the membrane to determine the sequence. The next generation, that replaces proteins with solid-state nanopores fabricated from synthetic materials, is now being tested in the field. “This is currently being evaluated in Sierra Leone for testing Ebola virus and is intrinsically better than current larger sequencing systems, but not yet ready for worldwide deployment”, commented Tim Peto, Co-Leader for the Infection Theme of the Oxford Biomedical Research Centre in the UK. It can also be applied to identify bacteria, as it is able to generate gene sequence data without having to culture a

sample first in the laboratory. “There is a huge effort now to get it working in cases where you haven’t got test facilities, where you want to do it on an uncultured sputum”, said Peto, referring to tuberculosis, an important health threat in developing countries. As he pointed out, molecular-level diagnostics requires advances not just in nanoscale technology but also the knowledge base to analyze sequence data. His group is involved with whole-genome sequencing of the TB mycobacterium on a machine called Illumina Miseq and the Nanopore MinION, which can generate both strain information for surveillance and antibiotic resistance data to determine the best treatment.

Another example of sequence-based pathogen diagnosis is the GeneXpert Omni system from Cepheid, based in California, USA. The US\$3,000 machine is suitable for smaller clinics and can conduct four tests simultaneously using a variety of samples, although the larger versions are only affordable or viable for major centers. As Peeling pointed out though, such systems are getting faster and cheaper, bringing them closer to the end point of care. “The GeneXpert now produces results in under an hour and there is quite a lot of hope that we can bring them down to all except village level”, she said. “They can run on batteries, so just need access to a source of charging”.

Other systems exploit microfluidic technology. One such device, developed by Colombia University in New York for testing for HIV and syphilis, is embedded in a small dongle powered by a smartphone, which can display the results and transmit them to a remote center [3]. The device replicates the mechanical, optical, and electronic functions of an enzyme-linked immunosorbent assay (ELISA) and can perform 96 tests on a single smartphone charge. It was used in a study by healthcare workers in Rwanda to test whole blood obtained by finger prick for HIV to prevent mother-to-child transmission.

There is more work needed though to improve the accuracy of small, portable devices, the sensitivity of which is in the range of 92–100% with specificity 79–100%. Lower sensitivity creates false negatives: A pathogen test with a 99% sensitivity would on average correctly identify 99 out of 100 people infected, while falsely ruling one out. Lower specificity in turn causes false positives: A 99% specificity would correctly

identify 99 out of 100 people as not carrying that pathogen, while falsely ruling one in. Both are key criteria for assessing the effectiveness of a diagnostic, although as Huggett pointed out, the relevant values will vary with the epidemiology or prevalence of the condition being tested for. “If a disease is incredibly rare and you test everybody, then a specificity of 99% becomes quite a low value”, he said.

Molecular diagnostics for HIV and prion diseases

By contrast, for a more prevalent disease such as HIV AIDS, a sensitivity of 99% will lead to many carriers of the virus being misdiagnosed. “Right now around 140 million rapid HIV tests are performed each year, and even if the error rate was only 1%—and we know it is much more than that—then there would be more than 1 million wrong results a year”, said Peeling. Despite this, the ability to conduct HIV tests quickly and easily in the field has helped to diagnose many patients far more quickly than before. Positive tests are followed up for confirmation anyway, and the challenge is to make the sensitivity as high as possible to avoid false negatives at the first hurdle.

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Given these requirements, some rapid HIV tests, such as the One Step HIV 1/2 Antibody Test from SD Bioline, claim sensitivity of 100%, with specificity of 99.8%. One Step is an immunochromatographic assay for the differential and qualitative detection of all antibodies specific to HIV-1, including subtype O and HIV-2 simultaneously, in human serum, plasma, or whole blood. This is a simple, low-tech procedure, similar to the common home pregnancy test.

While advances in molecular diagnostics have been most prominent for bacterial and viral infections, progress has also been made for detecting other infectious agents, including prions. The most recent advances are associated with a technique called

quaking-induced conversion (RT-Quick) seeding, which is capable of detecting minute amounts of the pathologic protein in cerebrospinal fluid or olfactory mucosa samples. In the RT-Quick assay, small amounts of infectious prions are added to normal protein to seed or cause the misfolding as seen in the disease. The assay is then quantitated by measuring serial dilutions of the samples and determining the loss of seeding activity.

RT-Quick is used to diagnose sporadic Creutzfeldt–Jakob disease (CJD), the most common form of human transmissible spongiform encephalopathy. Until recently, diagnosis began with a largely qualitative process, based on signs of dementia combined with at least two other clinical symptoms, including visual disturbances and ataxia. If this initial test was positive, definite confirmation required neuropathologic examination or detection of the CJD-specific abnormal prion. The new test is much simpler and has so far proved remarkably successful, achieving both 100% sensitivity and specificity on a controlled sample of 61 people with sporadic CJD and 71 without.

There are good reasons for making the test readily available, according to Jeremy Garson, from the University College London Hospitals NHS Foundation Trust in the UK. The first reason is the possibility of a second wave of variant CJD as a result of the original exposure to BSE-infected beef 25–30 years ago. This revolves around a common polymorphism at codon 129 of the normal PrP gene, which seems to govern susceptibility to prion disease according to whether methionine (M) or valine (V) is encoded. To date, all definite cases of variant CJD have occurred only in patients with the MM genotype, who are homozygous for methionine. However, a suspected case has been found of a person with the MV genotype contracting CJD, suggesting that such individuals might still be susceptible but have a long incubation period, as has already proved to be the case for other forms of acquired prion disease, such as kuru [4]. Garson argues that this makes an efficient, low-invasive test for CJD valuable to identify such cases early. The second motivation is to rule out other disorders, such as Alzheimer’s disease, that may present similar symptoms. “Accurate discrimination of prion diseases from other neurodegenerative illnesses or dementias is important because some of these alternative diagnoses are

treatable”, said Garson. “Accurate diagnosis of prion disease is also important to improve the reliability of patient selection in any future therapeutic trials of anti-prion agents”.

Rare genetic diseases

Another area of medicine in great need of molecular-level diagnostics is rare genetic diseases. Rare diseases are usually defined as affecting at most 1 in 2,000 people, but with 6,000–8,000 such diseases, they cumulatively affect more than 30 million people in the European Union, an incidence as high as 1 in 25 (<https://ec.europa.eu/research/participants/portal/desktop/en/opportunities/h2020/topics/sc1-pm-03-2017.html>). Moreover, rare diseases are hard to diagnose by their symptoms alone, with 25% of sufferers waiting 5–30 years for a diagnosis, with the initial one being wrong 40 % of the time [5].

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Diagnosis of rare genetic disease often starts with a referral to a clinical genetic service followed by laborious sequential testing for single gene variants when this seems indicated. However, the advent of the chromosomal microarray and parallel sequencing, either targeted at multiple variants simultaneously or the whole genome, has led to a marked improvement in diagnostic success [5]. These techniques are now being incorporated in various clinical programs for rare diseases, such as Australia’s Rare and Undiagnosed Diseases Diagnostic Service (RUDDS), which has been going since 2013. This is already revolutionizing diagnosis, according to Gareth Baynam, Head of the Western Australian Register of Developmental Anomalies, who is involved in RUDDS. “Currently the cause of approximately 50% of rare diseases has been identified and the great majority of these are genetic. We have seen a tripling of diagnostic rates in the last 12 months, trending towards a 40% diagnostic rate”, he said.

Baynam highlighted how diagnosis benefits from a combination of approaches: “For example the Undiagnosed Diseases Program Network and Network International blends human expertise in new paradigms with multiple technologies, genomic, epigenomic, metabolomic, model organisms systems, new consenting and data sharing processes”. He also cited the value of facial recognition since some rare diseases have characteristic visible symptoms susceptible to 2D or 3D computer image analysis (<http://www.crcsi.com.au/research/4-4-health/current-projects/4-412-cliniface/>). By way of example, fetal alcohol syndrome, arising when babies are exposed to excessive alcohol in the womb, causes distinctive facial features such as small eyes, a thin upper lip, and a smooth area beneath the nose.

Improved cancer diagnostics

Another major application for advanced diagnostics is cancer to test for genetic variants in tumor cells. One major difference from rare diseases is that many types of genetic alteration occur in cancer cells: single nucleotide variants; duplications, insertions, or deletions: changes in exons or gene copy number; large-scale duplications or deletions across entire genes; and structural variants at the chromosomal level. All of these can potentially be detected by molecular profiling through tests that vary from detecting single-point mutations in a given gene to whole-genome sequencing.

An example of the former is a test for a mutation commonly observed in melanoma that looks only for a specific nucleotide substitution at position 1799 of the BRAF gene. For more complex cancer diagnostics, a number of devices are becoming available based on next-generation sequencing (NGS), which promise to improve tumor profiling and lead to therapies better personalized to the individual, or at least the tumor concerned. These technologies are changing not just diagnostics but also the way tumors are classified, by enabling analysis of thousands of samples across many tumor types. This is resulting in the identification of new driver mutations, mutagenic patterns, and other features of tumor biology [6]. A major challenge now lies in integrating and analyzing all the data to relate the molecular features of cancer to biological and clinical characteristics.

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While the clinical benefits are still some way off, progress has been made toward a more fundamental requirement of cancer diagnostics which could almost be called a holy grail: a blood test for all forms of cancer that can be performed in principle at the end point of care. Such a test has been developed at the University of California, San Diego, and the inventors claim that it not only identifies cancer early on but also pinpoints the location without need for an invasive biopsy [7]. According to the senior author, Kun Zhang, Professor of Bioengineering at the UC San Diego Jacobs School of Engineering, the test was discovered almost by accident while looking for molecular signals from cancer cells. The team noticed that normal cells were also emitting signals and realized that this could yield a test for both presence and origin of an early tumor. The method involves screening for CpG methylation haplotypes in the genome. The pattern of this methylation determines gene expression and therefore underpins variation between cells, which helps to identify the tissue affected. Such a test could be employed as part of routine primary care screening, especially among older patients, who could then be referred for more sophisticated screening in the event of a positive result.

Metagenomics

There is also potential for identifying cancer at an early stage through application of metagenomics, which is another huge area for advanced diagnostics. Metagenomics involves sequencing of DNA obtained from environmental samples to identify pathogens and can be applied to analysis of gut microbiota in diagnostics. The key point is that various conditions are associated with characteristic signatures in the microbiota, measured primarily as relative abundances of different bacteria. One study successfully identified several species that could be used as taxonomic markers for detecting

colorectal carcinoma through metagenomic sequencing of fecal samples [8].

The power of metagenomics is the non-targeted scanning of all DNA molecules in a sample. Once acquired, the sequence can then potentially be interrogated for many questions, such as the presence of pathogens, metabolic pathways, antimicrobial resistance, overall community composition, or specific cancer signatures.

There is also potential for identifying complex phenotypes of intestinal and skin disorders that are not associated with a single pathogen but emerge as a result of complex ecological interactions within microbiota [9]. The main challenge lies in making sense of all the data and obtaining signatures relevant for diagnostics and treatment. For this to happen, more bioinformatics work is needed, not just in the development of novel tools and algorithms but also to assist application in the clinic. As sequencing technology continues to evolve rapidly, the main bottleneck for metagenomics, as well as genomic analysis in general, will thus lie in the ability to make sense of the data.

Partly for this reason, some in the field are skeptical over the diagnostic potential in the nearer term. A major issue is that metagenomics only gives access to a subset of the underlying molecular biology, according to Paige Lacy, Professor of Medicine and Dentistry at the University of Alberta in Canada. “The sequencing capability is certainly shooting far ahead of diagnostic understanding”, she said. “The problem is that there is so much more than DNA to the cell. [...] There is indeed a substantial need for bioinformatics to fill our understanding of all of the RNAs, proteins, and metabolites that fulfil the body’s intricate functions by application of transcriptomics, proteomics, and metabolomics, among many other omics

technologies”. Lacy also questions whether metagenomics can be applied successfully to complex metabolic conditions, because these are not always genetically driven. “For example, in diabetes, the loss of insulin secretion from the pancreas leading to the loss of glucose uptake by peripheral tissues may result from many different factors that are completely unrelated to genetics”, she explained.

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It will therefore require more work on all the associated multidisciplinary technologies, especially bioinformatics, to make the fundamental step forward from today’s diagnosis based on single biomarkers, such as glucose for type 2 diabetes or uric acid for gout. “These are single biomarkers that have been used successfully in clinical labs around the world”, Lacy said. “Our challenge is to apply complex metabolomics of plasma, urine, and other body samples to see if we can detect diseases or conditions that are difficult to detect using existing technologies. This would involve the analysis of multiple metabolites in these samples and to determine if there are patterns of these changing with exposure or disease incidence”. These same challenges apply to next-generation sequencing in general and thus circumscribe the growing optimism around molecular diagnostics for complex conditions.

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