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New Molecular Platforms for the Diagnosis of Invasive Fungal Infections

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The rapid escalation and high infection-related mortality of invasive fungal infections (IFIs) has prompted the need for rapid, more sensitive and accurate diagnostic tools. Much focus has been on the development of new molecular platforms that enable, broad range detection and identification of fungi from a variety of clinical specimens. However, the wider application of these methods is limited by the lack of standardisation, in part due to the variety of molecular methods. Although there is no universally accepted DNA detection system, three commercial *in vitro* diagnostic (IVD) assays have recently become available. These are generally faster than in-house assays, less laborious and rigorously maintained by high quality control standards, but are very expensive and still need to be run in parallel with 'gold standard' methods as clinical evaluations are ongoing.

Commercial systems

Several groups have evaluated the LightCycler® SeptiFast assay (Roche Diagnostics) for the detection of six fungal and 14 bacterial pathogens from whole blood specimens (covering 95% of the most frequently isolated organisms from blood culture) [1-7]. The system utilises dual internal transcribed spacer (ITS)-directed FRET probes and melting curve analysis to provide species identification within 6 h. The detection limit is 30 cfu/ml (100 cfu/ml for *Candida glabrata*) and is potentially more sensitive than blood culture [3]. Studies, however, have shown that the assay is more useful for the detection of bacterial, rather than fungal pathogens. Further validations are warranted to confirm its clinical and diagnostic utility.

The FXG™: RESP (Asp +) (Myconostica Ltd, Manchester, UK) system was released in 2008 and employs molecular beacons to detect *Aspergillus* and *Pneumocystis jirovecii* in respiratory samples. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) have been reported to be 74%, 93%, 76% and 92%, respectively, for *Aspergillus* detection; and 97.4%, 92.9%, 90.4% and 98.1%, respectively, for *P. jirovecii* detection [8, 9]. Microbial cross-reactions were not apparent (except against *Penicillium* sp.). Prospective and supportive clinical trials are ongoing to evaluate the clinical utility of this kit.

The third available IVD assay is repetitive-sequence-based PCR (rep-PCR; DiversiLab System, Spectral Genomics, Houston, TX). This platform uses primers that target non-coding repetitive sequence regions which are separated on a microfluidic chip (Aligent Bioanalyser 2100) that measures fluorescence intensity and migration time. Resulting fingerprints are compared with known standards, generating a percentage similarity (>85%) and cluster profile for identification. The current rep-PCR library holds patterns for species of *Aspergillus*, *Fusarium* and *Candida*, dermatophytes, *Blastomyces dermatidis*, *Coccidioides* and *Histoplasma capsulatum*. Numerous studies have demonstrated its potential [10-13], but the kit is restrictive as it can only be used on pure cultures and is dependent on an up-to-date rep-PCR library.

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Other novel systems

In addition to IVD platforms, there have been major developments in other molecular tools for the diagnosis of IFIs. Recent work in our laboratory has centred on the development of a novel multiplex-tandem PCR (MT-PCR) platform (AusDiagnostics, Alexandria, NSW) to identify up to 16 fungal pathogens from culture, blood culture, whole blood, serum and plasma specimens [14-16]. This system consists of two-rounds of amplification and melting curve analysis which is statistically compared to the expected melt temperature for species identification. Results are available within 2 h without need for DNA extraction from culture specimens. A retrospective analysis of candidemia patients found that the MT-PCR assay could detect *Candida* DNA in 74% of patients up to four days prior to the blood culture signalling positive [16]. Its use as a prospective screening tool for candidemia is yet to be assessed. The MT-PCR costs ~$14/sample and its automation is appropriate for laboratories with limited molecular experience.

The Luminex® multianalyte profiling (xMAP)™ system (Luminex Corp, Austin, TX) has also shown promise as a diagnostic tool since it has large multiplexing and high-throughput capability. This platform uses microsphere-based suspension array technology to simultaneously identify up to 100 probe hybridization reactions in a single reaction vessel within 1 h (up to 96 reactions can be performed concurrently). Fluorescence of captured amplicons is measured by flow cytometry. Luminex has been used to successfully identify *Candida*, *Fusarium*, *Aspergillus*, *Trichosporon*, *Malassezia* and *Cryptococcus* species from a variety of samples, including blood and tissue specimens [17, 18]. Sensitivity and specificity is 90-100% and 94.3-100%, respectively. The system is cheap ($5.70/sample); however, initial set-up costs are expensive and thus most suited for laboratories already equipped with a flow cytometer.

Isothermal amplification is another novel concept in diagnostic mycology. These platforms are robust, simple, cheap (~$2/sample) and are only dependent on a single incubation temperature for the generation of millions of target DNA copies. Such systems include nucleic acid sequence-based amplification (NASBA) [19, 20], loop mediated isothermal amplification (LAMP) [21-23] and rolling circle amplification (RCA) [24-26]. Each method is unique, employing catalytic enzymes, DNA strand displacement or circular DNA probes for amplification. Major fungal species have been identified from culture and clinical specimens with high sensitivity and specificity. Comparative studies between isothermal systems, real-time PCR and serological methods have shown similar results. These assays are well suited for general microbiology laboratories.

Finally, the concept of DNA barcoding has generated much excitement in recent years. Barcoding is based on the premise that accurate, automated species identification can be achieved by sequence analysis of short, standardised gene regions. Reference strains are used to generate standard sequence “barcodes”. Currently, the ITS region has been proposed as the default region for fungal species identification and work continues towards the assembly of a universal sequence-based identification system.

The availability of new molecular platforms has shown much promise in their application for early rapid diagnosis of IFIs with subsequent improvements in patient prognosis. The issue, however, of assay standardisation needs to be addressed. To this end, under the International Society for Human and Animal Mycology, the Laboratory Working Party of the European *Aspergillus* PCR Initiative is currently developing a protocol to standardise *Aspergillus* PCR methodology including specimen preparation, DNA extraction, PCR parameters and inclusion of appropriate controls. It is anticipated that molecular tools will be the future of diagnostic mycology. Comprehensive reviews of the major molecular methods have been published [27, 28].

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Brian O’Toole is an epidemiologist who has followed a peripatetic research path. He completed his PhD in psychology/neuroscience (Sydney, 1980) and a later Masters in Public Health by research (Sydney, 1990) into risk factors for mortality in 20-40 year old men. With major interests in research design and analysis, psychiatric epidemiology and the psychological effects of trauma, he has pursued largely a research career in community medicine, epidemiology, psychiatry and public health. Milestones have included headship of the only academic survey research centre in Australia; a major set of clinical epidemiologic studies in General Practice Integration research; a national evaluation study of early psychosis intervention programs; and a longitudinal study of the health of Australian Vietnam veterans and their families. He has developed and taught postgraduate courses in epidemiology since the mid-1980s and is a consultant to academic research groups in study design and statistical analysis strategies. He has over 100 published papers and government reports. His current purview at CIDM-PH is to assist staff and students with design and analysis of clinical epidemiological studies.

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