# Molecular Diagnosis of Invasive Fungal Disease

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I, Rebecca Louise Gorton, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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# Abstract

## Background

Invasive fungal infections (IFI) are opportunistic infections caused by yeast or filamentous fungi, typically presenting in immunocompromised patients (haemato-oncology, intensive care, HIV, solid organ transplant settings). This research aims to comprehensively evaluate molecular diagnostics to address the current shortfall in IFI diagnosis and, where appropriate, embed molecular methods into routine clinical service.

## Methods

Molecular methodologies, including PCR, MALDI-TOF MS and PNA-FISH, were evaluated on clinical sample cohorts from the Royal Free Hospital NHS Foundation Trust. Each method was critically appraised for: statistical performance, clinical utility and suitability for service adoption.

## Results

MALDI-TOF MS improved yeast agar culture identification, demonstrating 97.4% (185/190) concordance with ITS rRNA sequencing, and time to identification was significantly reduced ($p<0.01$, 24 hrs. v's 15 mins). Lower identification rates of 66% (33/50) were observed when applying MALDI-TOF MS directly to blood culture for yeast identification. In contrast PNA-FISH identified 98.5% (93/96, CI: 91.2, 98.9) of yeasts direct from blood culture within 30 minutes. Using PCP PCR a 60% (3/5) increase in the detection of PCP from BAL in non-HIV patients was demonstrated compared with GMS staining. Overall sensitivity was 100% (95% CI: 56.6, 100) and specificity was 97.9% (95% CI: 88.9, 99.6) for the diagnosis of PCP. *Aspergillus* PCR demonstrated a sensitivity of 100% (95% CI: 34.2, 100) and specificity of 93.8% (95% CI: 86.4, 97.3) for the diagnosis of IA but a low PPV of 28.6% (95% CI: 8.2, 64.1).

## Conclusions

Molecular diagnostic assays can improve the diagnosis of IFI through improved accuracy of identification and increased detection of fungal pathogens from specimens. Results must be interpreted alongside clinical presentation, as false positivity occurs utilising highly sensitive molecular assays. Dual biomarker strategies may improve the performance of molecular diagnostics but the associated impact on healthcare economics must also be considered

# Table of contents

## Abbreviations

| | |
|---|---|
| **A** | |
| AIDS | Aquired Immune Deficiency Syndrome |
| ARTEMIS DISK | Global Antifungal Surveillance Study |
| AST | Antimicrobial Susceptibility Testing |
| **B** | |
| BAL | Broncho Alveolar Lavage |
| **C** | |
| CDC | Center For Disease Control |
| CFU | Colony Forming Units |
| CLSI | Clinical and Laboratory Standards Institute |
| CNS | Central Nervous System |
| CSF | Cerbral Spinal Fluid |
| CT | Computerised Tomography |
| CVC | Central Venous Catheter |
| **D** | |
| DNA | Deoxyribose Nucleic Acid |
| DKA | Diabetic Ketoacidosis |
| **E** | |
| ECIL | European Conference On Infections In Leukemia |
| ECMO | Extracorporeal Membrane Oxygenation |
| EORTC MSG | European Organisation For Research And Treatment Of Cancer Mycoses Study Group |
| ESCMID | European Society Of Clinical Microbiology And Infectious Diseases |
| EUCAST | European Committee On Antimicrobial Susceptibility Testing |
| **F** | |
| FISH | Fluorescence *In situ* Hybridisation |
| **G** | |
| GI | Gastro Intestinal |
| GM-EIA | Galactomannan Enzyme Immuno Assay |
| GGO | Ground Glass Opacification |
| **H** | |
| HAEM | Haematology |
| HAART | Highly Active Anti Retroviral Therapy |
| HIV | Human Immunodeficiency Virus |
| HPLC | High Performance Liquid Chromatography |
| HSCT | Hematopoietic Stem Cell Transplantation |
| **I** | |
| IA | Invasive Aspergillosis |
| IC | Invasive Candidiasis |
| IDSA | Infectious Diseases Society of America |
| IFI | Invasive Fungal Infection |
| ITS | Intergenic Spacer Reigon |
| IV | Intra Venous |
| IFD | Invasive Fungal Disease |

**L**

| | |
|---|---|
| LOD | Limit of Detection |

**M**

| | |
|---|---|
| *m/z* | Mass To Charge Ratio |
| MALDI-TOF | Matrix Assisted Laser Desorption Isonisation Time of Flight |
| MIC | Minimum Inhibitor Concentration |
| mRNA | Messenger Ribose Nucleic Acid |
| MSG | Major Surface Glycoprotein |

**N**

| | |
|---|---|
| NPV | Negative Pedictive Value |

**O**

| | |
|---|---|
| ODI | Optical Density Index |

**P**

| | |
|---|---|
| PAS | Period Acid-Schiff |
| PCP | *Pneumocystis* Pneumonia |
| PCR | Polymerase Chain Reaction |
| PHE | Public Health England |
| PNA | Peptide Nucleic Acid |
| PPV | Positive Predictive Value |

**R**

| | |
|---|---|
| RNA | Ribose Nuclic Acid |

**S**

| | |
|---|---|
| SERS | Surface Enhanced Raman Spectroscopy |
| SMI | Standard For Microbiology Investigations |
| SOT | Solid Organ Transplant |

**T**

| | |
|---|---|
| TAT | Turn Around Time |
| TB | Tuberculosis |

**U**

| | |
|---|---|
| UK | United Kingdom |
| USA | United States Of America |
| UV | Ultraviolet |

## Table of figures

| | |
|---|---|
| | against a gold standard, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). |
| 1.20 | Diagrammatic representations of precision and accuracy. |
| 1.21 a) | Detection of amplification for conventional PCR (gel electrophoresis). |
| 1.21 b) | Real-time PCR (fluorescence detection in real time). |
| 1.22 | MALDI-TOF MS analysis demonstrating laser desorption of an analyte/matrix complex, with vaporisation into a vacuum chamber and time of flight of molecules based on their mass to charge (m/z) ratio through to a detector. |
| 1.23 a) | Phase contrast micrograph showing the round C. *albicans* and the oval-shaped C. *krusei* adhering to the endothelial cells. |
| 1.23 b) | The same section (as a) viewed by fluorescence microscopy. |
| 1.23 c) | Double exposure of the phase contrast and fluorescence micrographs. Note that only the round C. *albicans* cells, but not the longer C. *krusei* cells are stained by the fluorescently labeled probe 020. |
| 1.24 | Peptide nucleic acid (PNA) molecule bound to a DNA demonstrating the neutrally charged peptide substitution in the backbone replacing the negatively charged phosphate molecule. |
| 1.25 | Intracellular fluorescence *In situ* hybridisation using PNA probes complementary to ribosomal RNA molecules. |
| 1.26 | Routine Diagnostic Service at the Royal Free Hospital diagnostic microbiology laboratory – 2009. |
| **Chapter 2** | **General Methods** |
| 2.1 | Example of spectral matching with unknown spectra (top) matched against a reference spectrum (bottom). Peak matching and unmatched peaks are used to calculate the log similarity score. |
| **Chapter 3** | **Evaluation of solid agar culture processing methods and MALDI-TOF MS for the identification of clinically relevant yeast isolates** |
| 3.1 | Log similarity scores obtained for 190 yeast isolates processed by direct smear, on plate and conventional formic acid extraction. |
| 3.2 | MALDI-TOF MS spectrum obtained for a yeast isolate with a result of ‘no-identification’. The threshold for peak acceptance is indicated by the dotted line. Peak intensity and abundance indicates this MS spectra should have resulted in a successful identification if a matching spectra was present in the reference spectral database. |
| 3.3 | Diagrammatic representation of the yeast identification workflow in our laboratory. |
| **Chapter 4** | **Improving the diagnosis of Candidemia through the application of MALDI-TOF MS for the identification of yeasts direct from positive blood cultures** |
| 4.1 a) | Diagrammatic representation of Sepsityper processing with |

| | |
|---|---|
| | modifications indicated from A-F at the point of the process where they were introduced; increased blood volume. |
| 4.1 b) | Formic acid volume increase to 70µL. |
| 4.1 c) | Additional wash solution 2. |
| 4.1 d) | Water pre-wash. |
| 4.1 e) | Glass bead disruption of the pellet. |
| 4.1 f) | Vortexing of the blood culture bottle and 1mL aliquot. |
| 4.2 | Graph plotting average log similarity scores obtained from triplicate analyses of ten yeast blood cultures processed by Sepsityper with the addition of modifications: B = Increased formic acid to 70µL, C = Extra solution 2 wash, D = Pre water wash, E = Bead beating pellet, F = vortexing blood culture bottle and 1mL aspirate. Adjusted spectral analysis included cropping out the 0-4000kDa range from MS spectra. **p<0.01, *p=0.02, NS = no significant difference. Red lines indicate thresholds of >1.7 for genus identification and >2.0 for species identification. |
| 4.3 a) | Sections of MALDI-TOF MS spectra at acquisition for a) *Candida albicans* blood culture analysis with a log similarity score <1.6 indicating 'no identification'. |
| 4.3 b) | *C. albicans* blood culture with a successful identification with a log similarity score >1.8. |
| 4.3 c) | *Candida albicans* isolate identified by MALDI-TOF log similarity score 2.312. |
| 4.3 d) | Spectrum obtained from a negative blood culture by MALDI-TOF MS. The dotted line on both spectra indicates 600 absolute intensity. |
| **Chapter 5** | **Evaluation of MALDI TOF MS compared with conventional microscopic identification for the identification of filamentous mould isolates from clinical samples** |
| 5.1 | Example of an agarose gel for ITS rRNA amplicons. |
| 5.2 | Varying inocula (light, moderate, and heavy) for *Aspergillus fumigatus* fungal ball preparation for MALDI-TOF MS. |
| 5.3 | Box plot of log similarity scores obtained for four isolates (triplicate analyses) by MALDI-TOF MS using the pellet processing methods; a) drying in an incubator b) drying in a dry hot block c) no drying. Wilcoxon Signed Rank was used to calculate significance between the three methods. |
| 5.4 | Histogram displaying the distribution of log similarity scores obtained for 147 isolates processed by MALDI-TOF MS. Grey bars indicate correct species, white indicates the correct genus and black indicates incorrect identification. Dashed lines indicate manufacturers log similarity score thresholds for genus and species identifications. |
| 5.5 | Microscopic features for a) *Scytalidium hyalinum* and |

# Chapter 1 Introduction

## 1.1 Global incidence and mortality of invasive fungal disease

Invasive fungal infection (IFI) is a collective term used to describe a range of opportunistic infections typically presenting in critically ill, immunocompromised patients (Enoch *et al.*, 2017). The etiological agents may be either yeast or filamentous fungi. Patients at risk of IFI include those within haemato-oncology, intensive care, HIV, solid organ transplant settings and also patients who have undergone invasive surgery (Denning, 2005; Cooley *et al.*, 2014; Shao *et al.*, 2014; Alvaro-meca *et al.*, 2015; Harrison *et al.*, 2015; Enoch *et al.*, 2017). However, IFI is not exclusive to the respective patient groups and can present in many patients with immunosuppression. The definition of IFI is described as the isolation of a yeast or mould from sterile sites or deep tissues by culture and or the detection of fungal infection through histopathological examination of clinical specimens (Ascioglu *et al.*, 2002; De Pauw *et al.*, 2008)

The global burden of IFI is estimated to be in excess of 2 million cases per year globally (Brown *et al.*, 2012). In figure 1.1 the most frequently occurring fungal diseases are detailed with their estimated annual incidence and associated mortality rates (adapted from Brown *et al.*, 2012).

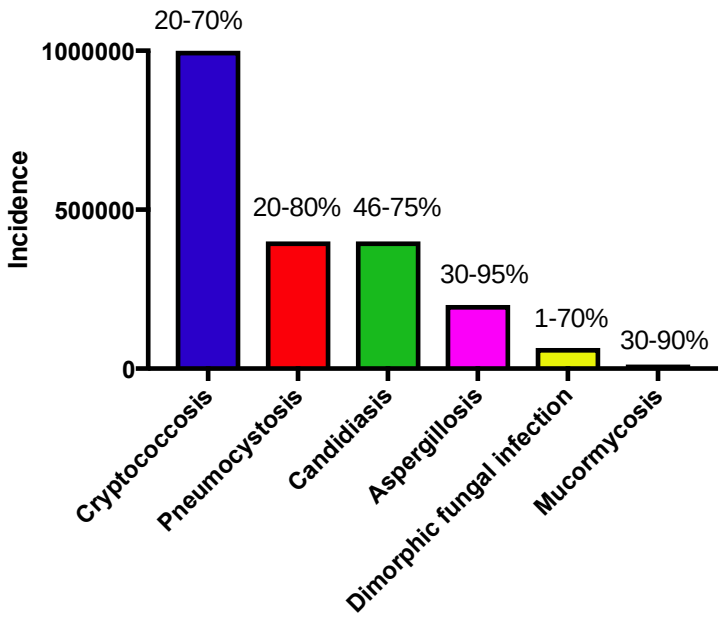



Figure 1.1 Histogram detailing the estimated global incidences of Invasive Fungal Infections per annum with associated mortality rates shown as percentage values (Brown *et al.*, 2012).

According to estimated data, the top four invasive fungal infections account for 96.4% of the total global burden and include; 1) cryptococcosis, accounting for approximately one million cases of life threatening infections per year (Park *et al.*, 2009; Maziarz and Perfect, 2016), 2) pneumocystosis accounting for 400,000 life threatening infections per year 3) candidiasis accounting for 400,000 life-threatening infections per year and 4) aspergillosis accounting for 200,000 life-threatening infections per year (Brown *et al.*, 2012). All IFIs are associated with significant morbidity and mortality rates ranging from 20-95% (Pfaller and Diekema, 2007; Baddley *et al.*, 2010; Brown *et al.*, 2012; Armstrong-James, Meintjes and Brown, 2014; Bitar, Lortholary, Strat, *et al.*, 2014; Kullberg and Arendrup, 2015). It is estimated that 1.5 million people die each year from IFI, with the top ten IFI accounting for more deaths per annum than TB or Malaria (Brown *et al.*, 2012).

The global incidence of IFI has increased over the last 30 years, as a consequence of the Human Immunodeficiency Virus (HIV) epidemic (Morse, 1995; Armstrong-James, Meintjes and Brown, 2014). The progression of HIV infected patients to development of acquired immunodeficiency syndrome (AIDS) allowed invasive fungal infections, including cryptococcosis and pneumocystosis, to present as opportunistic infections. The respective IFIs are recognised as AIDS defining diseases (Limper *et al.*, 2017). In recent years the long-term prognosis of HIV infection has dramatically improved with the development of highly active antiretroviral therapy (HAART) (Rouet *et al.*, 2006; Maggiolo and Leone, 2010; López-Sánchez *et al.*, 2015). In developed countries HIV is no longer a terminal disease but rather a chronic condition controlled with HAART and routine screening of CD4 T-cell counts and viral loads. In developing countries where HAART is not readily available the incidence of cryptococcosis remains high, with sub-Saharan Africa estimated to be carrying 75% of the global burden of cryptococcal meningitis (Ortblad, Lozano and Murray, 2013). In contrast, as HAART is the standard of care for HIV infected patients in developed countries it has been the catalyst for the dramatic decrease in the incidence of cryptococcosis. In the UK from 1996 to 2007, the incidence per 1000 persons in the UK fell from 3.0 to 0.2 (Garvey, 2011). In the UK estimates are now as low as <100 cases of cryptococcal meningitis diagnosed per annum (Pegorie, Denning and Welfare, 2017).

However, not all IFI have shown the same trend in decreasing incidence in the UK, this is due to non-HIV associated factors driving their sustained, if not increasing incidence (Warnock, 2007; Cuenca-Estrella *et al.*, 2008; Erjavec, Kluin-Nelemans and Verweij, 2009; Meis and Chakrabarti, 2009; Bajwa and Kulshrestha, 2013). By considering the patients who develop IFI it becomes clear that a key factor is the advancement in

medical practices including in-hospital and outpatient management of severe diseases such as organ failure, cancers and leukaemia. The resultant immune deficient host environment arising from aggressive immunosuppressive pharmacological strategies used in the treatment of severe disease is a major contributing factor to the proliferation of IFI (Warnock, 2007).

In the last twenty years advances in the treatment of haematological malignancies have included increasingly intensive chemotherapy regimens. As a consequence patients experience profound levels of immunosuppression for longer periods (Warnock, 2007). Advances in therapeutic techniques such as extracorporeal membrane oxygenation (ECMO), gastrointestinal surgery and solid organ transplantation have also improved the chance of survival for patients with life-threatening diseases but often patients require care in an the intensive care setting, and are by proxy susceptible to IFI (Gautam, 2006; Brown, Denning and Levitz, 2012; Gardner *et al.*, 2012). The use of corticosteroids in solid organ transplant patients is also associated with an increased risk of IFI (Rostaing and Malvezzi, 2016). The National Health Service (NHS) in the UK is a mature centrally managed health care system offering advance health care across the population; therefore IFI secondary to underlying diseases are expected to increase in incidence.

### 1.2 Overview of Invasive fungal infections in the United Kingdom

The incidence of IFI, other than cryptococcosis, in the UK is estimated to be much higher than 100 cases per annum. Invasive candidiasis (IC) was estimated at 4473 cases in 2013, for invasive aspergillosis (IA) the burden was estimated to be 2912 per year and 587 cases of *Pneumocystis* infection were estimated to have been diagnosed (Pegorie, Denning and Welfare, 2017). Current trends from UK clinical surveillance data indicate that the incidence of IFI is increasing (Pegorie, Denning and Welfare, 2017). However, there is a lack of robust multisite or national surveillance data to conclusively support this (Pegorie, Denning and Welfare, 2017). From voluntary reporting to Public Health England (PHE), the UK government body for monitoring national trends in infection, the reported rate of candidemia increased in the year 2015-2016 from 2.7 per 100,000 to 3.1 per 100,000 population (PHE, 2016). Data published for 2016 shows a further increase to 3.5 per 100,000 population (PHE, 2017). The isolation of *A. fumigatus* and *Fusarium* sp from clinical specimens in the UK is detailed in figure 1.2. A marginal upward trend is described for both species from 2011-2015 but rates again remain low at 3 per 100,000 population for *Aspergillus* and 1.6 per 100,000 for *Fusarium* sp (Public Health England, 2016)

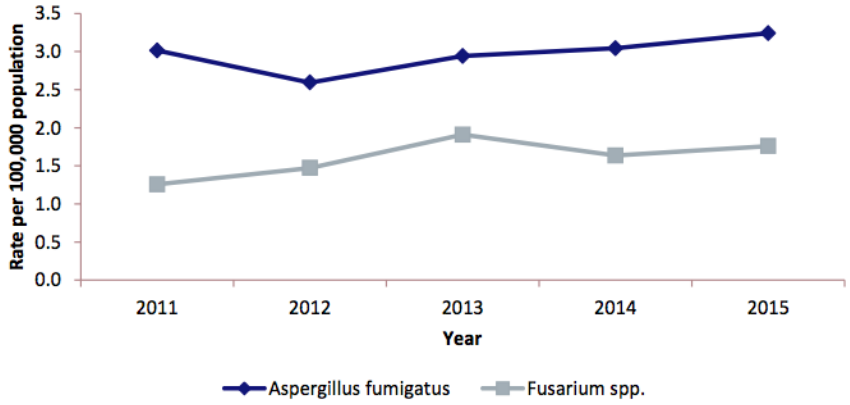



Figure 1.2 Frequency of isolation of *Aspergillus* and *Fusarium* species in the UK from 2011-2015. Data published by Public Health England.

As the incidence of IFI is low in comparison to other infectious diseases the question posed is why there is a need to improve the diagnosis of IFI? In short, there are limitations in diagnostic testing for IFI and IFI are high mortality rates. This combination is in turn driving the overuse of antifungal drugs for the prophylaxis and treatment of IFI. The overuse of antifungal drugs in healthcare is additionally associated with a significant economic burden (Wilson *et al*., 1998, Desai, Cavanaugh and Guo, 2011).

In 1998 Wilson *et al* reported the estimated total direct cost of systemic fungal infection in the USA to be $2.6 billion per annum and the average attributable cost per-patient was $31,200 (Wilson *et al*., 1998). A proportion of this cost is due to the overuse of antifungal therapies within the tertiary care setting. From September 2002 to September 2003 the annual hospital antifungal expenditure in the UK was £52.5M ('Fungal Diseases in the UK', 2006). Recent estimates in antifungal spend for the year 2013 were £112M (PHE, 2015); more than doubling the amount of expenditure over a 10-year period. This trend is not unique to the UK health care system, with reports from the USA demonstrating a ten-fold increase in the utilisation of antifungal agents for IFI between 2001 and 2009 (Desai, Cavanaugh and Guo, 2011). The economic impact of fungal infection is high and measures to reduce the unnecessary use of antifungals is required, not only for expenditure management but also to preserve their efficacy

### 1.3 Overview of Antifungal drugs

Current therapeutic choices for invasive fungal disease are limited to four classes of antifungal agents (Denning and Hope, 2010; Campoy and Adrio, 2017). Table 1.1 details the current armamentarium of antifungals and their cellular or molecular targets.

From the outset it is important to recognise that fungi are eukaryotes, with structural features including membrane bound organelles, a nucleus and they may be unicellular or multicellular in form (Vellai *et al*., 1999). Therefore, fungi are similar in structure to mammalian cells. The targets for antifungal agents are molecules that are similar in structure to those found within human cells. This makes the treatment of IFI challenging as antifungal therapies can be associated with host cellular toxicity (table 1.1) (Denning and Hope, 2010). When choosing an antifungal regimen several factors require consideration. Antifungal agents, such as fluconazole have a narrow spectrum in their activity profile, whilst agents such as amphotericin B (Ambisome®) have a broader spectrum (Denning and Hope, 2010). If the causative fungus has not been identified this prohibits the use of narrow spectrum antifungal agents. Establishing both the source and site of infection is important as not all antifungals can be distributed to all sites within the body; more specifically the echinocandins are unable to penetrate the central nervous system (CNS), gastrointestinal (GI) and renal tract (table 1.1) (Denning and Hope, 2010; Campoy and Adrio, 2017). The toxicity profile of antifungal agents also requires consideration.

Using liposomal amphotericin B (Ambisome®) in renal patients is challenging as this antifungal agent may contribute to additional renal damage. The drug ergosterol is similar in structure to cholesterol in mammalian cells (Laniado-Laborín and Cabrales-Vargas, 2009). Amphotericin B (Ambisome®) can bind to cholesterol causing cellular damage (Milhaud *et al.*, 2002). Using triazoles in HIV patients and patients receiving treatment for tuberculosis is also contraindicated due to the administration of drugs such as rifampicin which induce the cytochrome P450 pathway, significantly reducing the efficacy of triazoles (Brüggemann *et al.*, 2009). 5-flucytosine is contraindicated in haematology patients due to a toxicity profile that includes bone marrow suppression (Navarro and Senior, 2006). In addition to chemical composition, the formulation of the antifungal also must be considered. Until 2014 posaconazole was only available in oral formulation therefore, could not be administered to patients unable to receive oral preparations (McKeage, 2015). Patients well enough to be treated as an outpatient may have to be admitted to hospital as often the only antifungal option is in an intravenous formulation (Brüggemann *et al.*, 2009). Tailoring the correct antifungal for an infection in the right patient requires expertise. Clinical mycologists with such expertise are lacking in number within healthcare, therefore clinicians rely heavily on nationally steered published guidelines (often re-interpreted as local policy) to direct their choice of antifungal therapy.

**Table 1.1 Antifungal drug classes for the treatment of invasive fungal disease, detailing mode of action, antifungal activity, toxicity, resistance mechanisms, formulations and penetration profile (adapted from Campoy and Kanafani 2017, Li *et al*., 2015).**

| Drug class | Mode of action | Drug name | Antifungal spectrum | Toxicity | Formulations | Resistance | Organ distribution |
|---|---|---|---|---|---|---|---|
| **Triazoles** | Inhibition of ergosterol synthesis, Cytochrome P450 pathway | Fluconazole | *Candida*, *Cryptococcus*, dimorphic fungi | Dose related: high levels associated with hepatotoxicity, renal toxicity, arrhythmia, and rash | Oral and Intravenous | Efflux pumps, several point mutations including: *ERG11, cyp51A* and *cyp51B* | Good penetration including CNS |
| | | Itraconazole | *Candida* and *Aspergillus* | | | | |
| | | Voriconazole | Broad spectrum including yeasts, *Aspergillus*, *Fusarium*, *Scedosporium*, *Mucorales* | | | | |
| | | Posaconazole | | | | | |
| | | Isavuconazole | | | | | |
| **Polyenes** | Disruption of the fungal cell membrane | Nystatin | *Candida*, *Cryptococcus*, *Aspergillus* and *Fusarium* | Not used systemically | Topical | Rare, *ERG3* defects | Good penetration including CNS |
| | | Liposomal Amphotericin B (Ambisome®) | Broad spectrum including yeasts, *Aspergillus*, *Fusarium*, *Scedosporium*, *Mucorales* | Kidneys, liver | Intravenous | | |
| **Echinocandins** | Fungal cell wall synthesis (1-3)-β-D-glucan | Caspofungin | *Candida* and *Aspergillus* | None | Intravenous | Poorly understood - Mutations in *Fks1* gene | Does not penetrate GI tract, Renal and CSF. Good for biofilms |
| | | Micafungin | | | | | |
| | | Anidulafungin | | | | | |
| **5-flucytosine** | Inhibits nucleic acid synthesis | 5-flucytosine | *Candida* and *Cryptococcus* | Bone marrow suppression | Intravenous | Common in monotherapy, mutations in cysteine deaminase | Good penetration including CNS |

## 1.4 Guidelines for the definition, diagnosis and management of invasive fungal disease

International guidelines provide the backbone for the clinical management of IFI. Evidence-based guidance defines criteria for diagnosing and treating patients suspected of having IFI. In the past decade four major groups (including two scientific societies) have published guidelines and criteria including: the European Society of Clinical Microbiology and Infectious Diseases (Ullmann *et al.*, 2012; Cornely *et al.*, 2014) and the Infectious Diseases Society of America (IDSA) (Pappas *et al.*, 2015; Patterson *et al.*, 2016) the European Conference on Infection in Leukaemia (ECIL) (Tissot *et al.*, 2016) and the European Organisation for the Treatment and Research of Cancer (De Pauw *et al.*, 2008) Table 1.2 summarises, in brief, the grading used for recommendations made within the respective guidelines. Recommendations are produced first by evaluating the evidence base, grading the quality of the evidence on the level the study. In principle randomised control trials are rated as high quality, well-designed clinical trials are considered moderate quality and low quality evidence is considered to be expert opinion, observational and clinical case studies. The quality of the evidence base is paired with a 'strength of recommendation', calculated from principles including: quality of evidence, the balance between benefit/harm and burdens, resource and cost and also patient's values and preferences (Ullmann *et al.*, 2012; Cornely, Cornely et al., 2014;, Cuenca-Estrella, *et al.*, 2014; Pappas *et al.*, 2015; Patterson *et al.*, 2016; Tissot *et al.*, 2016). Recommendations for diagnosis and treatment of IFI will be considered throughout chapter 1 alongside the specific IFI, with reference to the strength and grade of the recommendation as detailed in table 1.2.

**Table 1.2 Grading utilised by guidelines for recommendations made for the diagnosis and treatment of invasive fungal disease.**

| ***Strength of recommendation*** | | | ***Quality of evidence*** | | |
|---|---|---|---|---|---|
| **ECIL** | **IDSA** | **ESCMID** | **ECIL** | **IDSA** | **ESCMID** |
| A = Good | Strong | A= Strong | I ≥ 1 RCT | High | I ≥ 1 RCT |
| B = Moderate | Weak | B = Moderate | II ≥ 1 WDCT | Moderate | II ≥ 1 WDCT |
| C = Poor | | C = Marginal | III = expert opinion | Low | III = expert opinion |

RCT = randomised controlled trial, WDCT= well designed clinical trial

Where guidelines provide recommendations on treatment and diagnosis, the EORTC/MSG criteria provides a strategy by which patients can be classified into categories of proven, probable and possible IFI based on clinical presentation, underlying immunosuppression and pathological findings from the investigation of clinical specimens. The EORTC/MSG criteria published standard definitions for IFI for clinical and epidemiologic research (De Pauw *et al.*, 2008). These definitions were developed to facilitate the identification of reasonably homogeneous cohorts of patients (De Pauw *et al.*, 2008). Clinical trials designed to evaluate new drugs; diagnostics and patient management strategies also use the EORTC/MSG criteria to analyse data. The criteria also help to standardise analysis across studies internationally allowing communication between international researchers (De Pauw *et al.*, 2008). For the purpose of this research the EORTC/MSG criteria will be considered in chapter 9.

## 1.5 Overview of Immunity to fungal infection

The innate and adaptive immune system protect against IFI (Jiang, 2016). Physical barriers of the skin and mucosa provide non-specific protection (Jiang, 2016). If fungi invade past skin and mucosal barriers the specific innate and adaptive immune response is activated. Circulating white blood cells provide the innate response including neutrophils, macrophages, and dendritic cells (Jiang, 2016) that phagocytose and inactivate infecting fungal cells. If fungi evade this first line of defence the adaptive immune response is activated and T-cells (th1, th2 and th17, t-Reg) and B-cells secrete an array of cytokines and antibodies to target fungal pathogens (Jiang, 2016). The scope of the immune response to fungi is complex and breaches the scope of this research however; there are key immune components that are classified as risk factors when diagnosing fungal infection.

### Skin and mucosal barrier integrity

The skin and mucosal barriers protect hosts from fungal infection. *Candida* are commensal organisms of the gastrointestinal tract (Ohmit *et al.*, 2003; Southern *et al.*, 2008). In healthy individuals colonisation is without consequence as protective mucosal barriers prevent translocation of *Candida* into the blood stream. A breakdown in the integrity of the mucosal barrier, through surgery or chemotherapies, is a primary risk factor for invasive candidiasis (Moyes and Naglik, 2011; Netea *et al.*, 2015). Another major risk factor for IC is through a breach in the skin by the insertion of central venous catheters (CVC) and other intra-venous (IV) lines used for the administration of

treatment. In mucoraceous fungal infections skin/soft tissue trauma or trauma to the gastrointestinal tract can predispose patients to invasive infection (Spellberg, Edwards and Ibrahim, 2016; Ghuman and Voelz, 2017). Wounds become colonised from fungi in the environment and subsequent IFI develops. The ingestion of mucoraceous fungi with food sources, such as fruit and vegetables, in patients with damage to the integrity of the GI tract is also a risk factor for developing mucoraceous IFI, although the latter is a rarely occurring infection (Spellberg, Edwards and Ibrahim, 2016; Ghuman and Voelz, 2017). If fungi evade protective barriers and invade sterile sites immune cells are recruited to the site of infection, through signalling molecules inflammatory cytokines, chemokines and complement factor, to destroy the invading fungi (Eberyl and Davy, Immunology.org). When considering patients at risk of IFI the role of neutrophils is crucial for protection against fungal infection.

**Neutrophil role in the innate immune response to fungi**

Neutrophil cells are the first immune cells recruited to site of inflammation during fungal infection (Eberyl and Davy, Immunology.org). Fungi are engulfed by neutrophils through the process of phagocytosis. Fungal cells are internalized in a phagosome inside the neutrophil where antimicrobial molecules and reactive oxygen species bind to the phagasome and damage and/or kill fungal cells (Diamond and Clark, 1982; Levitz and Farrell, 1990; Hohl and Feldmesser, 2007; Dagenais and Keller, 2009). *In vitro* studies have demonstrated this killing action of neutrophils against *Aspergillus* hyphae (Diamond and Clark, 1982; Levitz and Farrell, 1990). In *Candida* infection neutrophils are crucial for early recognition and clearance of the fungus, but also prevent the morphological switch from yeast to filamentous forms (Duggan *et al*, 2015). In *Fusarium* infection studies have demonstrated damage to hyphae, primed by gamma interferon and granulocyte stimulating factor, through neutrophil attack (Gaviria *et al*., 2017). In mucroaceous mould infection increased neutrophil recruitment in the nasal tissues has been demonstrated in animal models (Ghuman and Voelz, 2017). Neutrophils and soluble mediators also play a role in facilitating clearance of PCP during infection (Gigliotti and Wright, 2005; Krajicek, Thomas and Limper, 2009; Kelly and Shellito, 2010). Therefore, neutrophils are crucial for the prevention of fungal infection. Having low levels of neutrophils (neutropenia) is a primary risk factor for developing invasive fungal disease. In particular prolonged periods of neutropenia are associated with an increased risk of fungal disease. Factors that reduce neutrophil levels and function include myeloablative chemotherapy, steroid therapy, myelodysplastic syndromes, HIV infection, sepsis and severe autoimmune conditions.

Whilst prolonged neutropenia is recognised as a primary risk factor of IFI, and is included in the EORTC definitions of IFI (de Pauw *et al*., 2008), overall defects in a patients' immune system is a key predisposing factor for development of IFI. The type of IFI a patient develops is co-associated with the patients underlying disease and mode of immune suppression.

### 1.6 Invasive fungal infections

IFIs can be broadly divided into two clinical categories: invasive yeast and invasive mould infections. The morphology and cellular structure of the two different forms of fungi facilitate the identification the infection resulting from the different fungal pathogens. Before attempting to diagnose IFI it is crucial to understand the clinical features of each disease (White, 2017). Pathogenesis and risk factors associated with different IFI allow for the selection of specimens most suitable for investigation. Considering what tests are available for a given disease and the performance characteristics of specific methods determines how specimens are prepared for analysis. It is important to appreciate that the choice of specimen itself introduces variables into the diagnostic process (White, 2017). The optimal specimen type will be determined by the disease manifestation or testing strategy employed.

### 1.7 Invasive Candidiasis

*Candida spp* are unicellular organisms that replicate asexually via the process of budding (Calderone and Fonzi, 2001). Some species, such as *Candida albicans* can switch from a unicellular state to a complex multicellular filamentous organism, this feature is thought to have developed to convey an opportunistic evolutionary advantage compared with other yeast species (Calderone and Fonzi, 2001). Figure 1.3 demonstrates a) unicellular blastoconidia of yeasts, b) pseudohyphae formations of tubular filaments c) hyphae with septa. The morphological characteristics of yeasts allow for dispersion through the blood stream, as unicellular blastospores in fungemia, but also dissemination into deep-seated organs where some yeast species then invade tissues forming multifocal lesions in the hyphal form (Calderone and Fonzi, 2001).

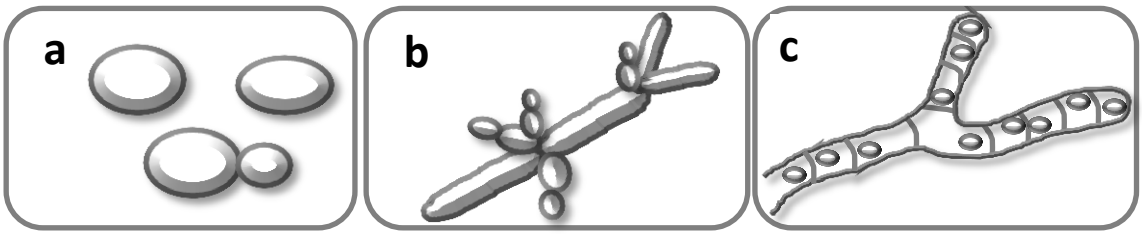



Figure 1.3 Morphological characteristics of yeasts, as a) blastospores b) pseudohyphae and c) true hyphae/mycelia.

*Candida* are also very competent at forming biofilms within tissues and on plastics that become heavily resistant to antifungal agents (Vila and Rozental, 2016). The biofilm starts with adhesion of blastospores to a surface that then develops, through hyphal production into a mature biofilm surrounded by extracellular matrix. Daughter cells are then dispersed from the biofilm into the surrounding environment, fig 1.4 (Vila and Rozental, 2016)

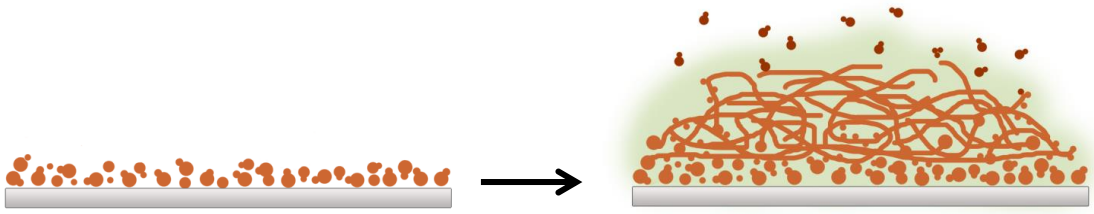


Figure 1.4 *Candida* adhesion and biofilm formation through hyphal production and extracellular matrix (Vila and Rozental, 2016).

Invasive candidiasis is a well-recognised nosocomial infection affecting hospitalised patients in developed countries (Pfaller *et al.*, 2011; Kaye *et al.*, 2014; Wisplinghoff *et al.*, 2014). Mortality is high, reaching up to 40% in some settings despite the administration of antifungal therapy (Kullberg and Arendrup, 2015). With increasing incidence of IC new insights into diagnostic and therapeutic strategies are required to improve the incidence and outcome of IC.

### 1.7.1 Epidemiology of invasive candidiasis

Invasive candidiasis incidence rates in the general population are very low. Recent surveillance data reports incidence rates of 2.9-6.3 per 100,000 population in the UK (Odds *et al.*, 2007; Oeser *et al.*, 2014) and 10.4 per 100,000 populations in Denmark

(Arendrup *et al.*, 2011). *Candida* is reportedly the fourth most common cause of blood stream infection (BSI) in the United States however, source data is mainly derived from ICU where patients are at a higher risk of IC (Pfaller *et al.*, 2001). When focusing on the specific patient groups at risk of IC, incidence rates increase one hundred-fold, to an incidence of 2-6.9 per 1000 patients in the intensive care setting (Eggimann, Bille and Marchetti, 2011; Kett *et al.*, 2011). Other patient groups at higher risk of IC include haemato-oncology, surgical and other immunocompromised patient groups such as HIV/AIDS (Fraser *et al.*, 1992; Ascioglu *et al.*, 2002; Arendrup *et al.*, 2010; Ullmann *et al.*, 2012)

Several risk factors are associated with IC. Mucous membrane colonisation of the GI tract is a risk factor in immunosuppressed patients. The fungal burden is higher than normal due to overgrowth of *Candida* as the bacterial flora is reduced due to broad spectrum antimicrobial therapy (Fraser *et al.*, 1992; Alonso-Valle *et al.*, 2003; Pauw *et al.*, 2008; Arendrup, 2010). Gastrointestinal surgery and chemotherapy-induced injury to the lower gastrointestinal tract are also major risk factors for IC (Calderone and Fonzi, 2001; Dellinger, Levy and Rhodes, 2013; Wisplinghoff *et al.*, 2014). Damage to the protective integrity of the gut mucosal surface allows colonising *Candida* to translocate into tissues and the bloodstream resulting in IC (Janum and Afshari, 2016). Once translocation has occurred into the bloodstream *Candida* is capable of forming biofilms on plastic surfaces. Vascular catheterisation is a significant risk factor in the development of Candidemia as the catheter becomes the direct source of infection (Janum and Afshari, 2016). The extremes of age also present a risk as the patient's immune system is either underdeveloped in neonates or is declining in the elderly (Arendrup, 2013; Kaye *et al.*, 2014). Parenteral nutrition is also a risk factor for IC, mainly in neonates, but most likely reflects the severity of the patient's condition rather than being a true source of IC (Widmer *et al*., 1997). It is often the case that a patient will experience a combination of the respective risk factors prior to developing IC.

Although Candidemia is the most common presentation, deep-seated infection of tissues such as the liver, spleen and kidney can also arise from dissemination during blood stream infection (Clancy *et al* 2013). Both candidemia and deep seated candidiasis can present in isolation or overlap as a consequences of each other (Clancy and Nguyen, 2013; Kullberg and Arendrup, 2015). Disseminated candidiasis, in the absence of blood stream infection, presents a diagnostic challenge. Recent advancements in radiological techniques are allowing for more accurate diagnosis. Figure 1.5 demonstrates micro abscesses in the liver and spleen due to *Candida*

infection (Cornely *et al*, 2015). These *Candida* lesions are non-specific as differential diagnoses could be lymphoma of the liver, leukemic infiltrates or metastases (Cornely, Bangard and Jaspers, 2015). Despite radiology being non-specific positive imaging can be used to prompt further pathological investigations. Chronic disseminated (hepatosplenic) candidiasis can occur in patients at risk of IC with prolonged neutropenia (Cornely, Bangard and Jaspers, 2015). Chronic infection of the blood stream can also lead to more complicated infections of sites such as ocular infection or infection of the central nervous system (Calderone and Fonzi, 2001; Kullberg and Arendrup, 2015). Disseminated invasive *Candida* infections are more commonly associated with virulent species of *Candida* such as *C. albicans* and *C. tropicalis* (Calderone and Fonzi, 2001; Kullberg and Arendrup, 2015) but several species have been implicated in invasive disease (Deorukhkar, Saini and Mathew, 2014).

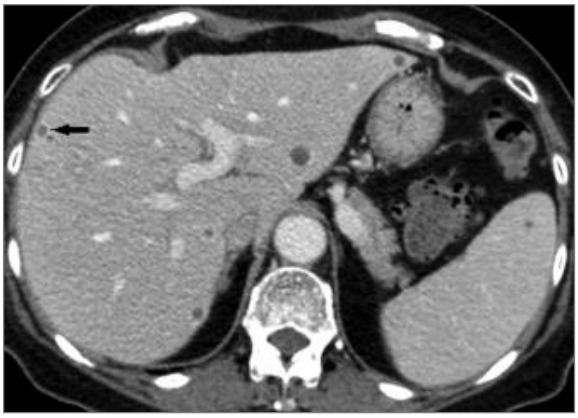


Figure 1.5 CT scan with multiple hypodense *Candida* micro abscesses in the liver and spleen. The arrow indicates peripheral contrast uptake of the small abscess (Cornely, Bangard and Jaspers, 2015).

The distribution of species implicated in IC globally has remained relatively consistent with 90-95% of all candidemia attributed to five main species (Kullberg and Arendrup, 2015). *C. albicans* remains the primary etiological agent (Tortorano *et al.*, 2006; Pfaller *et al.*, 2011; Guinea, 2014; PHE report, 2016) with C*. parapsilosis*, *C. tropicalis, C. glabrata and C. krusei* making up the five top species implicated in IC (Pfaller *et al.*, 2011; Kullberg and Arendrup, 2015). Some species are more prevalent in certain clinical settings, for example in the haematology setting the incidence of *C. krusei* is relatively high causing 12-16% of candidemia (Pfaller, Diekema, *et al.*, 2008). Recent surveillance data from Denmark has highlighted a shift towards increased incidence of azole resistant *Candida* species (Arendrup *et al.*, 2011) which has been associated with the prophylactic use of azole antifungal agents such as fluconazole (Hope, Morton

and Eisen, 2002). The increasing incidence of azole-resistant species is a key factor for driving guidelines to recommend echinocandins as empirical therapy for candidemia (Ullmann *et al.*, 2012; Pappas *et al.*, 2015; Tissot *et al.*, 2016). It is important to note that a small proportion of IC cases are caused by rare non-*Candida* yeast species, e.g. *Blastoschizomyces capitatus* and *Trichosporon sp*. Guinea (2014) reported, from the ARTEMIS DISK Global Antifungal Surveillance Study, a decrease in incidence of *C. albicans* with increasing incidence of 'other' yeast species implicated in fungemia from 1997-2007, as shown in figure 1.6 (Guinea, 2014).

Therefore, determining the species of yeast implicated in IC is of increasing importance in order to guide antifungal therapy. Until the species has been determined, treatment of invasive candidiasis relies on broad-spectrum antifungal agents as empirical therapy (Garey *et al.*, 2006; Farmakiotis *et al.*, 2015). As improved outcome for patients is reliant on administering the correct antifungal agents, early diagnosis is essential.

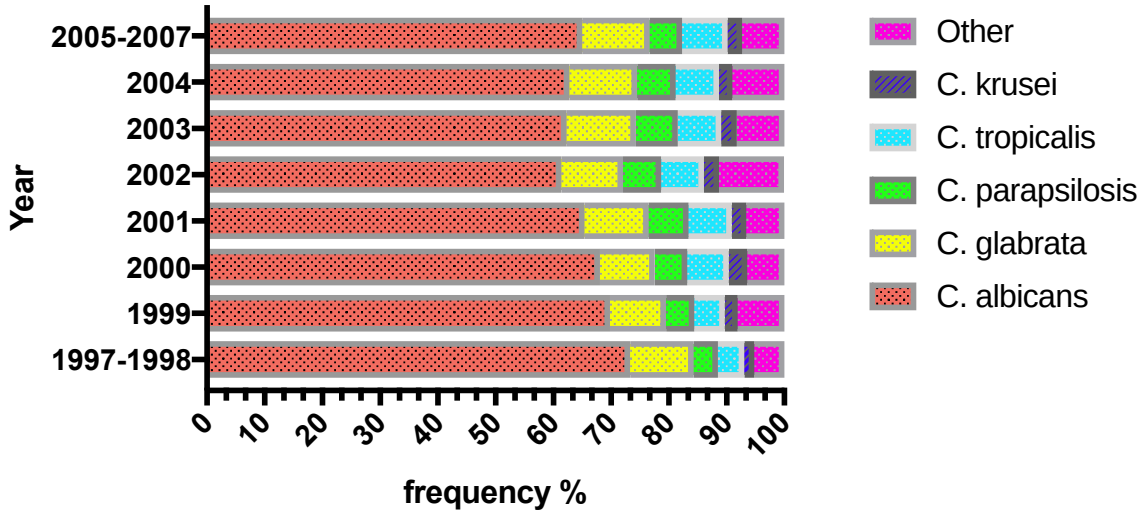



Figure 1.6 Frequency distribution of *Candida* species isolated from candidemia (adapted from Guinea, 2014).

### 1.7.2 Clinical presentation of invasive Candidiasis

Invasive candidiasis presents with non-specific symptoms such as pyrexia and signs of sepsis, which is often undistinguishable from bacterial sepsis (Hollenbach, 2008; Bassetti *et al.*, 2017). Suspicion of IC usually arises when a patient's fever is refractory to broad-spectrum antibacterial therapy. As a result, candidemia and other forms of IC

can go undiagnosed for 3-4 days before being suspected and follow-up diagnostic investigations are initiated. Despite the administration of antifungal therapy, chronic disseminated candidiasis and intra-abdominal infection often present as refractory infection as the source of infection can be difficult for antifungal drugs to penetrate and often requires source control through drainage or surgical intervention (Cornely, Bangard and Jaspers, 2015). Chronic *Candida* infection can often be associated with chorioretinitis which presents as blurred vision of floating black spots caused by *Candida* fungal balls within the eye (Lashof *et al.*, 2011; Kauffman, 2015). Chorioretinitis is associated with poor outcome if diagnosed and occurs in 8-16% of patients with Candidemia (Shah *et al.*, 2008; Clancy and Nguyen, 2013). *Candida* endocarditis is rare, accounting for less than 2% of all endocarditis cases. However, *Candida* endocarditis can occur in patients with risk factors such as valvular prosthesis, congenital heart disease, history of infective endocarditis and other prosthetics such as pace makers (Kauffman, 2015). In a review of 44 patients who developed candidemia with prosthetic heart valves 16% had evidence of endocarditis at the time of presentation and 9% developed endocarditis after candidemia (Melgar *et al.*, 1997).

### 1.7.3 Laboratory diagnosis of Invasive Candidiasis

#### 1.7.3.1 Blood cultures

The diagnosis of IC is heavily dependent on laboratory investigation. The current gold standard for the diagnosis of IC is a positive blood culture sample, despite being associated with a low test sensitivity of only 21-75% (Kami *et al.*, 2002; Thorn *et al.*, 2010; Clancy and Nguyen, 2013). The limitation of blood culture for the diagnosis of IC is in the poor detection of deep-seated disseminated candidiasis where blood cultures are often negative (Clancy and Nguyen, 2013). Despite this lack of sensitivity blood culture is still recommended as an essential investigation for IC (Schelenz *et al.*, 2009; Pappas *et al,*. 2016). If a patient is suspected to have IC current guidelines recommend daily collection of blood cultures, aspirating a total volume of 40-60mL to be distributed across 4-6 bottles in 10mL aliquots to optimise the sensitivity of the culture process (Pappas *et al.*, 2015). Additional blood cultures should be collected during febrile episodes to maximise the chance of recovering yeasts from blood stream infection. Improved performance has been shown with the inclusion of Mycosis bottles into the blood culture pathway (Chiarini *et al.*, 2008) but they are not routinely used in clinical practice. Most laboratories in the developed world incubate blood cultures on automated blood culture systems providing an electronic positive flag. Although

automated systems have been shown to be more effective in the speed of recovery of microorganisms from blood cultures (Wilson *et al.*, 1999) there is still a delay in the time to diagnosis, reportedly on average 25.9 hours for *Candida* (±24.9h) (Lai *et al.*, 2012).

The availability of other clinical specimens such as sterile fluids and tissue biopsies from patients with suspected IC relies heavily on the patient's condition and therefore invasive specimens are not commonly taken. If tissue and fluid samples are collected the standard of care is to perform microscopy and culture on specialised fungal media (Schelenz *et al.*, 2009).

#### 1.7.3.2 Microscopy and culture

When a blood culture is detected as positive or when performing direct microscopy on a specimen the most important diagnostic test used is the Gram's stain from which yeasts can be detected (Thairu, Usman and Nasir, 2014), figure 1.7. Yeasts stain Gram positive, owing to the capture of the crystal violet between the cell wall layers as it complexes with iodine (Henrici, 1914). In 1919 the scientist Arthur T Henrici described his studies on the staining characteristics of yeasts declaring ‘’*So far as I know, all of the yeasts are Gram-positive, and they stain so deep and solid a black that it seemed to me not unlikely that they would retain the stain even more firmly than the ordinary Gram-positive bacteria*’’ (Henrici, 1914).

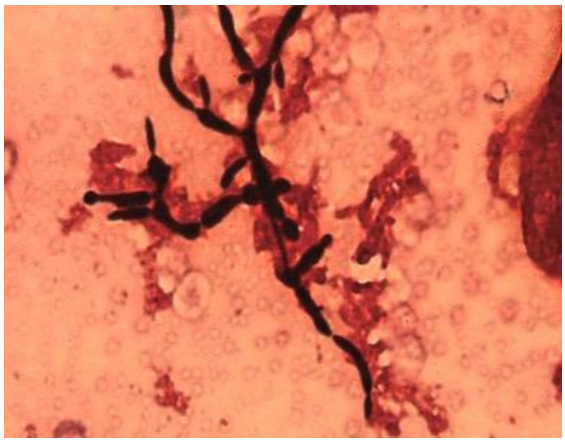


Figure 1.7 *Candida* sp stained Gram Positive (Gorton *et al*., 2015).

To this day Gram's stain still provides a rapid result available to clinicians in less than 15 minutes providing valuable information for targeted management of patients with antifungal agents. Gram's stain is limited to generalising the identification to that of 'yeasts seen' (Beveridge, 2001). The next step in the diagnostic pathway is to subculture the yeast onto specialised media, either *Candida* chromogenic media or

Sabouraud's agar which requires an additional incubation time of at least 18-24 hours to culture yeasts for identification (Ainscough and Kibbler, 1998).

The clinical microbiology laboratory is required to identify yeast cultures to species level when isolated from patients with suspected IC (Schelenz *et al.*, 2009). If chromogenic media are used for subculture *C. albicans* can be identified as apple green colonies with a high degree of accuracy of >99% (Odds and Bernaerts, 1994). For non-albicans species the conventional method of yeast identification is phenotypic testing through biochemical profiling with a variety of assimilation reactions of carbohydrates such as glucose and lactose (Davey *et al.*, 1995; Sheppard *et al.*, 1998; Romney *et al.*, 2000). *Candida* cultures grown on cornmeal agar supplemented with Tween 80 are performed alongside biochemical assays for the characterisation of morphological characteristics such as hyphae, pseudohyphae and chlamydospores (figure 1.8). After an additional 24-48 hours incubation the reaction profile can be determined as positive or negative through colorimetric or turbidity readings alongside the cornmeal agar morphology (Davey *et al.*, 1995; Sheppard *et al.*, 1998; Romney *et al.*, 2000). The resulting profile/score is then matched to a database and the most likely identification assigned (Davey *et al.*, 1995; Sheppard *et al.*, 1998; Romney *et al.*, 2000).

The most utilised commercial assays are the Auxacolor system (Sanofi Diagnostics Pasteur, Paris, France), the Vitek 2 system (Biomerieux, Marcy-l'Etoile France) and the API *Candida* (BioMerieux, Marcy-l'Etoile France). In a recent meta-analysis of assay performance, across 26 published studies, the accuracy of three biochemical identification systems' were reportedly 89% for the Auxacolor, 80% for the API ID32C, and 93% for the Vitek 2 (Posteraro *et al.*, 2015).

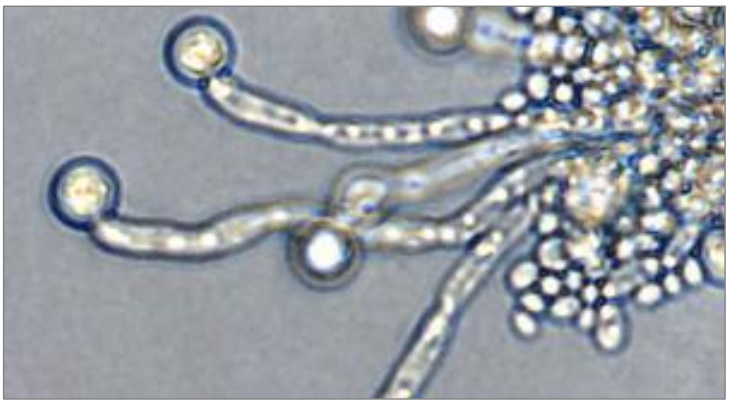


Figure 1.8 Cornmeal agar culture of *Candida albicans* demonstrating chlamydospore production and true hyphae (Hardy Diagnostics).

Misidentifications were most common with rarer yeast species as they were either not included in the assay databases or could not be distinguished from profiles of more commonly isolated species (Posteraro *et al.*, 2015). In total, the identification pathway can equate to a turnaround time of 4 days before the identification can be reported.

#### 1.7.3.3 Antifungal susceptibility of *Candida*

With accurate species identification it is possible to predict the susceptibility of *Candida* species to antifungal agents. A global survey published in 2011 reported 98% of *C. albicans* isolates from IC infection measured susceptible to fluconazole (Pfaller *et al.*, 2011). Susceptibility to fluconazole was lower for *C. tropicalis* (91%) and *C. parapsilosis* (93.2%) isolates but still remained high above 90% (Pfaller *et al.*, 2011). In contrast identification of C*. glabrata* or *C. krusei* from IC would contraindicate the use of fluconazole with a high proportion *C. glabrata* (31.3%) being resistant resistant to fluconazole and *C. krusei* being intrinsically resistant (Pfaller *et al.*, 2011). Therefore, accurate species identification can contribute to the antifungal management.

Diagnosrtic guidelines recommend that antifungal susceptibility testing (AST) should be performed on all yeasts isolated from sterile sites or blood cultures (Schelenz 2012). The minimum inhibitory concentration (MIC) of yeasts can be determiend using gradient MIC strips (Barnes *et al.*, 1996) or microdilution methods (Arendrup *et al*., 2017). For the interpratation of MIC breakpoints it is essential to obtain an accurate species identification (Espinel-Ingroff *et al.*, 1999; Cantón *et al.*, 2005; Pfaller, Chaturvedi, *et al.*, 2008). Susceptibility breakpoints for *Candida* species are different dependent on the species therefore, misidentification of isolates can in turn result in misinterpretation of susceptibility results (Borman *et al.*, 2012). This is currently a limitation for laboratories using biochemical methods to identify yeasts from blood cultures (Posteraro *et al.*, 2015).

With up to 88.9% of candidemia patients reportedly receiving inappropriate antifungal therapy for 24 hours or more until culture and AST results are known reducing the time to identification and increasing the accuracy of identification could have a significant clinical impact on morbidity and mortality of IC through the administration of appropriate targeted antifungal therapy (Sutepvarnon *et al.*, 2008; Arnold *et al.*, 2010; Zilberberg *et al.*, 2010).

#### 1.7.3.4 (1-3)-β-D-glucan and PCR for the diagnosis of invasive candidiasis

Non-culture techniques including PCR and serological testing for the detection of fungal antigens have the potential to improve the diagnosis of invasive candidiasis (Clancy and Nguyen, 2013). The detection of DNA or antigens from specimens may aid in the diagnosis of 40% of invasive candidiasis that is undetected by blood culture (Clancy and Nguyen, 2013).

PCR is a rapid technique, reducing the TAT to results by an average of 2.2 days compared to culture and provides a species level identification as much as 4 days earlier than culture based techniques (Lau *et al.*, 2010). However, use of PCR for candidemia is limited with only select clinical services choosing to adopt this method for diagnosis of IC. The main prohibiting factor preventing clinical diagnostic services from developing PCR for invasive candidiasis is assay sensitivity, as less than 1 cfu/mL is known to be present in blood during candidemia infection (Pfeiffer *et al.*, 2011). Large blood volumes are required for extraction to enable detection. Reichard *et al (*2012) recently reported intra-laboratory inconsistency when detecting *Candida* from blood using PCR, and the limit of detection determined to be between 10 and 100 cfu/ml (Reichard *et al.*, 2012). The use of *Candida* PCR is therefore still experimental or utilised in highly specific clinical scenarios and by specialist service laboratories with experienced mycologists.

Antigen detection, specifically the detection of (1-3)-β-D-glucan, is becoming more frequently utilised to assist in the diagnosis of IC. In the last decade (1-3)-β-D-glucan has become an established biomarker for the investigation of invasive fungal infection. (1-3)-β-D-glucans are a group of β-D glucose polysaccharides contained within the cell wall of most fungi (excluding mucoraceous fungi) (Brown and Gordon, 2003; Synytsya and Novák, 2013) plants and bacteria. Many fungi produce (1-3)-β-D-glucan and during the infection process it is possible to detect the antigen within serum. Two commercially available assays, the Fungitell (Cape Cod) and Fungus (1-3)-β-D-glucan assay (Dynamiker, Tianjin) tests are available and have shown comparable performance (White *et al.*, 2017). Due to the commonality of the target across species positive results are non-specific and do not differentiate between fungal infections at the species level. A recent meta-analysis reported a pooled sensitivity and specificity of 78% and 81% respectively for the diagnosis of IC (He *et al.*, 2015). Improved performance was noted in a systematic review by ECIL in 2012 when two consecutive positive tests were used for diagnosis with a sensitivity and specificity of 49.6% and 98.9% respectively (Lamoth *et al.*, 2012). The PPV and NPV was predicted to be

83.5% and 94.6% respectively, at a disease prevalence level of 10%, which is high compared to actual prevalence rates in the UK and Europe (Lamoth *et al.*, 2012). The PPV and NPV are likely to be reduced in lower prevalence settings (Bentley, Catanzaro and Ganiats, 2012). The performance of (1-3)-β-D-glucan assays for the diagnosis of IC is limited as a stand-alone test but is a useful negative screen for several fungal infections.

#### 1.7.3.5 Prophylaxis and treatment of invasive candidiasis

In table 1.3 recommendations from the three major guidelines are compared for the treatment of invasive candidiasis; ECIL (2016), IDSA (2016) and ESCMID (2012) (Tissot *et al.*, 2016, Pappas *et al.*, 2015; Ullmann *et al.*, 2012). Encouragingly all guidelines appear to consistently recommend the same approach to the management of IC in both general and haemato-oncology patients. The empirical therapy of choice is an Echinocandin, with step down to fluconazole after 5 days of therapy if an azole susceptible species is identified. For *C. krusei* voriconazole is recommended as a step down alternative and for *C. parapsilosis* isolates fluconazole is suggested as a more effective alternative due to reports of high MIC to echinocandins (Ullmann *et al.*, 2012; Pappas *et al.*, 2015; Tissot *et al.*, 2016). For clinically stable patients where the species is known to be azole susceptible fluconazole can be used empirically, but is only recommended in this specific scenario. The length of treatment is 2 weeks after clearance of candidemia, determined by a negative blood culture (Ullmann *et al.*, 2012; Pappas *et al.*, 2015; Tissot *et al.*, 2016). The removal of central venous catheters (CVC) and indwelling lines is recommended as early as possible. For disseminated *Candida* infection Liposomal Amphotericin B is recommended with the inclusion of 5-flucytosine for ocular or CNS infection (Ullmann *et al.*, 2012; Pappas *et al.*, 2015; Tissot *et al.*, 2016).

Prophylaxis in high-risk patients is usually through the administration of fluconazole, which in guidelines received a weak recommendation; moderate-quality evidence from the IDSA (Mossad, 2004; Uko *et al.*, 2006; Weitkamp *et al.*, 2008; Springer *et al.*, 2012; Eschenauer *et al.*, 2015; Pappas *et al.*, 2015). In neonates non-absorbable nystatin is recommended as an oral agent for decolonisation of the GI tract but also oral fluconazole to be assessed on a case by case basis (Hope *et al.*, 2012).

**Table 1.3. Comparison of recommendations from guidelines for the management of invasive candidiasis; ECIL, IDSA and ESCMID**

| | | ECIL 2017 (Tissot *et al.*, 2016) | | IDSA (Haematology) (Pappas *et al.*, 2015) | | | | ESCMID (Ullmann *et al* 2012) | |
|---|---|---|---|---|---|---|---|---|---|
| **Intervention** | **Recommendation** | **General** | **Haem** | **General** | | **Haem** | | **General** | **Haem** |
| First line empirical | Echinocandin | AI | AII | Strong | High | Strong | Moderate | AI | AI |
| Alternative empirical | Liposomal AmpB (Ambisome) | AI | AII | Strong | Moderate | Strong | Moderate | BI | AI |
| Non-critical patient once species is known | Fluconazole | AI | CIII | Strong | High | Weak | Low | - | - |
| Step down >5 days | Fluconazole | AI | CIII | Strong | High | Weak | Low | BII | ns |
| *C. krusei* step down | Voriconazole | BI | CIII | Strong | Low | - | - | - | - |
| Catheter removal | Rapid removal | AII | BII | Strong | Moderate | - | - | AIII | AII |
| Length of antifungal treatment | 2 weeks after clearance | - | - | Strong | Moderate | Strong | Low | BII | BI |
| Chronic disseminated candidiasis | Liposomal AmpB or an echinocandin with fluconazole step down | - | - | Strong | Low | - | - | BII | - |

### 1.8 *Pneumocystis* pneumonia (PCP)

*Pneumocystis jirovecii* is the etiological agent responsible for an acute, severe pneumonia in immunosuppressed patients, known as *Pneumocystis* pneumonia or PCP. Prior to the advent of whole genome sequencing *P. jirovecii* was thought to be protozoan but was reclassified as a yeast after whole genome sequencing aligned *P. jirovecii* with the fungal kingdom (Frenkel, Bartlett and Smith, 1990). *P. jirovecii* is unique as a fungus with a multiphasic life cycle that occurs within the alveolar space of the host, figure 1.9 (www.tulane.edu). The cystic form of *Pneumocystis*, namely the ascus, is circular or ovoid in shape and measures 4–7 μm in diameter (Aliouat-Denis *et al.*, 2009; Skalski, Kottom and Limper, 2015). A thick outer wall made of abundant (1-3)-β-D-glucan contains ascospores that mature and are released from the ascus through a small pore. The *Pneumocystis* cells then mature to become the more metabolically active trophic forms of *P. jirovecii* (Finkelman, 2010; Skalski, Kottom and Limper, 2015). In the infective process the trophic cells are the replicative form of *P. jirovecii* which, unlike other yeast species that replicate via asexual budding; replicate through the process of binary fission (Aliouat-Denis *et al.*, 2009; Skalski, Kottom and Limper, 2015).

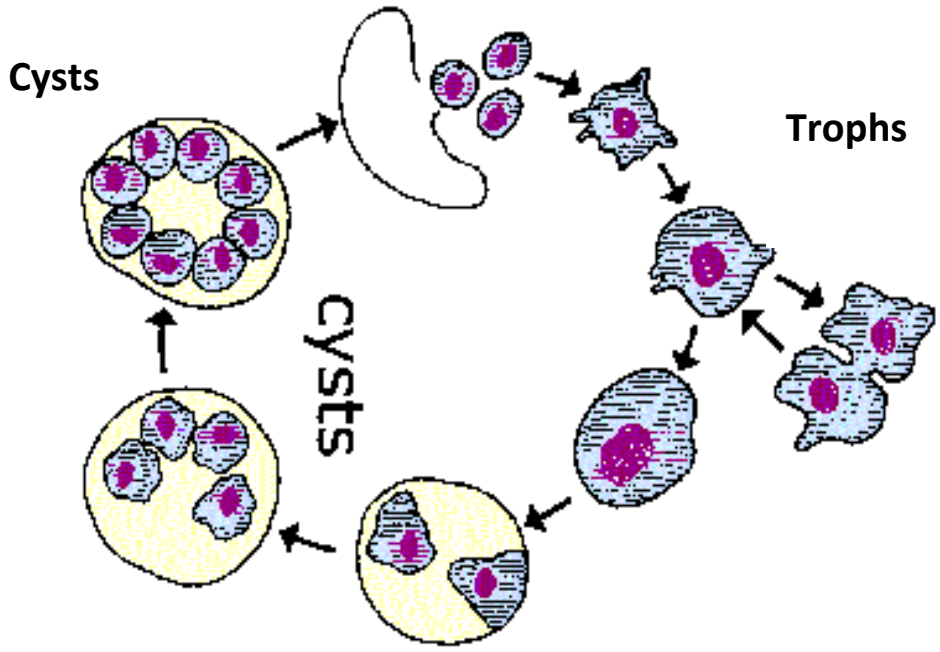



Figure 1.9 The replicative life cycle of *P. jirovecii* showing ascus formation, troph maturation and release with replication through binary fission (www.tulane.edu).

### 1.8.1 Epidemiology of *P. jirovecii* infection

*Pneumocystis jirovecii* is distributed worldwide and is known to colonise the lungs of healthy individuals, with up to 75% of children testing sero-positive by the age of 4 years old (Vargas *et al.*, 2001; Respaldiza *et al.*, 2004). Transmission of *P. jirovecii* from person to person has been documented (de Boer *et al.*, 2007; Yazaki *et al.*, 2009). More recently hospital acquired infection has been described in outbreaks on renal and ICU units (Chapman *et al.*, 2013; Robin *et al.*, 2017). Genotypically distinct mixtures of organisms have been reported in more than 90% of cases, reinforcing the hypothesis that *P. jirovecii* is consistently inhaled from different environmental sources over time (Alanio *et al.*, 2017). Patients at risk of developing PCP were, until recently, mainly HIV infected patients, with PCP presenting as an AIDS associated disease. In HIV positive patients PCP is associated with 15-30% mortality rates (Helweg-Larsen, 2004; Teshale *et al.*, 2007). In the last decade, with the advent of HAART therapy and cotrimoxazole prophylaxis, the incidence of PCP in the HIV positive population has decreased significantly. Increasing incidence of PCP infection is reported in patients with acute and chronic leukaemia, dexamethasone treated patients (such as those with brain tumours), other corticosteroid treated patients (especially SLE, Wegener's, glomerulonephritis), transplant recipients who are not receiving cotrimoxazole, hypogammaglobinaemia and those patients on anti-TNF therapy (Yale and Limper, 1996; Pareja, Garland and Koziel, 1998; Enomoto *et al.*, 2010; Bitar, Lortholary, Le Strat, *et al.*, 2014). In the respective patient groups mortality is reported to be higher than in HIV positive patients, in some reports >50% mortality is described (Yale and Limper, 1996; Pareja, Garland and Koziel, 1998; Enomoto *et al.*, 2010; Bitar, Lortholary, Le Strat, *et al.*, 2014)

### 1.8.2 Clinical presentation of PCP

*P. jirovecii* pneumonia typically presents as a severe respiratory infection, which may precipitate rapidly into life-threatening pneumonia. The classical presentation of a patient with PCP is low-grade fever, a dry cough and dyspnoea. When listening to the lungs, through auscultation, fine crackles are common at presentation in patients with PCP. Oxygen saturation is typically low with patients describing drowning sensations and severe difficulty in breathing. It is important to acknowledge that this presentation is classical for a HIV positive patient presenting with PCP (Tasaka, 2015). In non-HIV patients' low-grade respiratory infection is indistinguishable from the presentation of other atypical respiratory infections. Clinical diagnosis in HIV-negative patients is more

challenging as PCP can be more acute and fulminant with high grade dyspnoea, fever, and chills (Catherinot *et al.*, 2010; A. Alanio *et al.*, 2016; Cordonnier *et al.*, 2016). HIV-negative patients are more likely to require mechanical ventilation (Pathak *et al.*, 2012). Table 1.4 details the patient groups that are associated with *Pneumocystis* infection and (seperatley) the therapeutic agents associated with a risk of infection (Catherinot *et al.*, 2010; Hayes and Denning, 2013; Roux *et al.*, 2014; Tasaka, 2015; A. Alanio *et al.*, 2016; Cordonnier *et al.*, 2016).

**Table 1.4. Clinical conditions and therapeutic agents associated with PCP**

| Conditions associated with PCP | Therapeutic agents associated with PCP (mode of immune suppression) |
|---|---|
| HIV | Corticosteroids (T-lymphocyte suppression) |
| Hematological malignancy | Alkylating agents such as cyclophosphamide) |
| Solid tumors | Chemotherapeutics (e.g. methotrexate) (DNA, RNA, proteins) |
| Autoimmune disease | TNF inhibitors (such as infliximab) |
| Solid organ transplant | Rituximab (targets CD20; B cells) |
| Severe combined immune deficiency | Cyclosporine (decreases lymphocyte function) |
| Hyper IgM syndrome | Azanthioprine (transplant immunosuppressant) |
| Wegener's granulomatosis | Sirolimas/tacrolimas (intracellular lymphocyte messaging) |
| Inflammatory bowel disease | - |
| Collagen vascular disorders | - |

As health care advances there is an ever-increasing range of therapies with varying mechanisms of immunosuppression that predispose patients to PCP infection. Targeted diagnosis and treatment of PCP is challenging and many disciplines within primary healthcare may be affected.

### 1.8.3 Radiological diagnosis of PCP

Radiological investigation of PCP can be highly specific if classical presentations are observed. Chest X-rays will display bilateral peri-hilar interstitial infiltrates (or ground glass opacification), described as having the appearance of 'bats wings' infiltrates (Hardak, Brook and Yigla, 2010), (as shown in figure 1.10 a). Radiology may not always present with typical features of PCP during infection. Figure 1.10 b demonstrates a patient with a right sided pneumothorax, which was diagnosed to be

due to *P. jirovecii* infection (Mu *et al.*, 2016). In early infection patients' radiological investigations (mainly X-ray) may be negative.

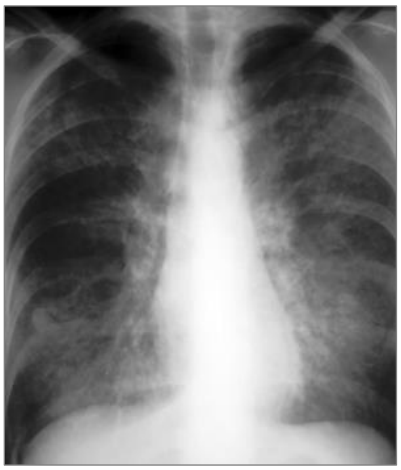
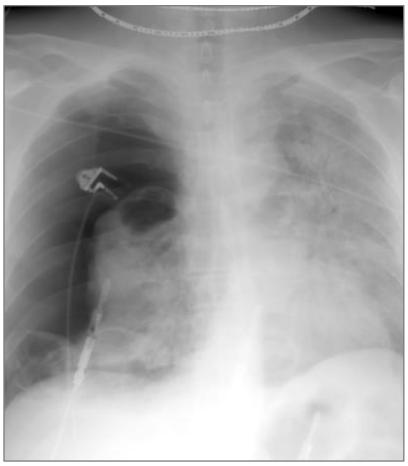
a)  b) 

Figure 1.10 a) Bilateral diffuse ground glass opacification in PCP b) Atypical presentation of a right side pneumothorax in PCP (Mu *et al.*, 2016).

A CT scan may be required to observe features of infection. Mu *et al* described the varying presentations at different stages of disease progression, detailed in table 1.5. Chest CT detected progression of disease earlier than X-ray (Mu *et al.*, 2016).

**Table 1.5 Radiological presentations in PCP patients on X-ray and CT at early, mid and late stages of infection (adapted from Mu *et al.*, 2016)**

| Stage of PCP | Manifestation (bilateral) | |
|---|---|---|
| | **CXR** | **Chest CT** |
| **Early** | Normal | Diffuse GGO |
| **Mid** | Infiltrates | GGO/patchy consolidations |
| **Late** | Consolidations | Predominant consolidations |

GGO = ground glass opacification, CXR = chest X-ray

### 1.8.4 Microscopy for the detection of *P. jirovecii*

*Pneumocystis* is non-cultivable in the diagnostic laboratory and highly specialized manipulation in lung cell lines is required for isolation (Schildgen, Mai and Khalfaoui, 2014). The current gold standard for diagnosis of PCP is direct visualization of *Pneumocystis* cells using microscopy. Historically several stains have been utilised including; toluidine blue O stain, the Gram-Weigert stain and Grocott's adaptation of Gomori's methenamine silver nitrate (MSN) stain (Newman, 1964). The toluidine blue

O stain and Gram-Weigert stain have several limitations; staining the cyst form only whilst also staining the background material, or co-staining other cells such as yeasts and red blood cells, making it difficult to distinguish *P. jirovecii* (Newman, 1964). Grocott's MSN stain shows the greatest contrast between the organism and its environment. Staining the cyst dark brown/black, as demonstrated in figure 1.11, and the background material light green, it is considered to be the best stain for identifying *P. jirovecii* and has remained the stain of choice to date by cytologists.

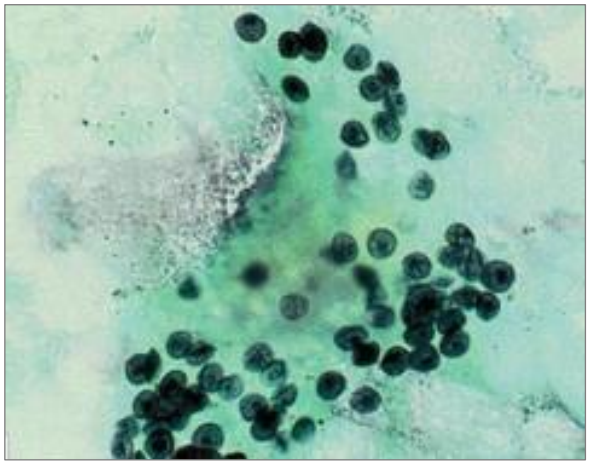


Figure 1.11 Grocott's staining of *P. jirovecii* in a broncho-alveolar lavage sample from a HIV-infected patient presenting with PCP (http://medicine.academic.ru/140233).

Despite demonstrating high specificity, the sensitivity of Grocott's MSN stain is highly variable ranging from 53% to 83.5% (Cruciani *et al.*, 2002a), influenced by sample type with detection from BAL being more sensitive than induced sputum (Silva, Bazzo and Borges, 2007). In our centre Grocott's MSN stain is utilised for the detection of *P. jirovecii*. An internal audit demonstrated that the provision of this service was affected by the working hours of our cytology department with a median turnaround time of 7 days (range 3-16) from the sample being taken to provision of results (in-house vertical audit, 2014). As PCP often requires rapid medical intervention, patients were almost always diagnosed clinically with radiological assistance. Grocott's MSN stain results served to confirm or rule out infection after treatment had been administered some days earlier.

An alternative technique to Grocott's MSN stain is a direct immunofluorescent (IF) method utilising fluorescein-conjugated monoclonal antibodies targeting *P. jirovecii* (Procop *et al.*, 2004). This stain is commercially available in several formats (Hauser *et al.*, 2011; Tia *et al.*, 2012; Choe *et al.*, 2014) and is used mainly in laboratories with a specialist mycology service. When compared with Grocott's staining IF is more

sensitive (Arasteh *et al.*, 1998) with a meta-analysis reporting sensitivities ranging from 79-94.4% (Cruciani *et al.*, 2002). The advantage of IF is that it can detect both cystic and trophic forms of *Pneumocystis*. The fluorescent signal, demonstrated in figure 1.12, most likely contributes to increased detection of *P. jirovecii* at lower concentrations. IF can also be performed in microbiology laboratories, making out-of-hours testing possible.

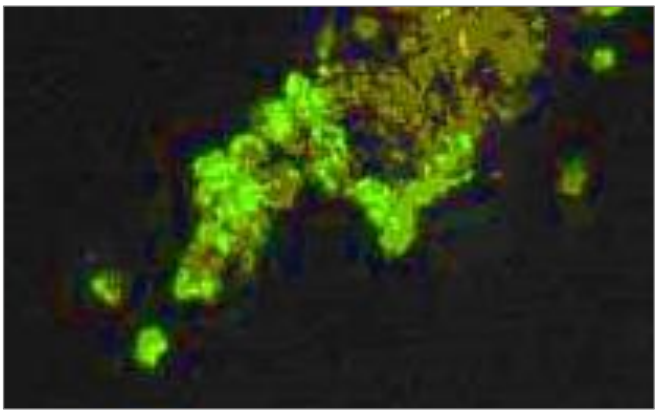


Figure 1.12 Immunofluorescence staining for the detection of *Pneumocystis jirovecii* in BAL fluid (Weinstein., 2014).

However, implementing the immunofluorescence-based assay requires careful consideration. Processing samples can be time-consuming, and analysis can be complicated by nonspecific background staining or auto-fluorescence from structures such as red blood cells or eosinophilic granules which if misinterpreted can lead to false positive results (Procop *et al.*, 2004). When considering immunofluorescence as a diagnostic assay there is a requirement to have a fully trained health care scientist to perform processing and microscopic analysis. There is also a need to consider the limit of detection when using fluorescence microscopy for *P. jirovecii,* reportedly 1.5 x $10^3$ cfu/mL from BAL (Mühlethaler *et al.*, 2012). This LOD may not be sensitive enough to diagnose PCP in the atypical patient groups presenting with symptoms of PCP. Specific patient groups including: haemato-oncology, renal transplant and those with complex immunological disorders are at risk of *P. jirovecii* infection, typically present with low grade disease (Bollée *et al.*, 2007; Roux *et al.*, 2014). In the respective patient groups the burden of *P. jirovecii* in infection has been shown to be lower than that of HIV patients (Hauser *et al.*, 2011; Seah *et al.*, 2012). Sample type can also affect sensitivity. The most frequently utilised specimen for diagnosis of PCP is broncho-alveolar lavage (BAL), which involves irrigating patient's lungs with 60-200mL of saline to sample the alveolar lung spaces (Svensson *et al.*, 2017). This inevitably has a dilution effect on the cfu/mL of organisms within the sample, which may further affect

the sensitivity of detection using microscopy. To give context, the significance of microorganisms such as *Streptococcus pneumoniae* in infection is considered to be upwards of $>10^6$ cfu/mL from a sputum whereas BAL utilises a cut off $>10^3$ cfu/mL which is accepted as significant from direct culture of the specimen (SMI B55, 2015). Utilising microscopy for the diagnosis of PCP may lead to infections in non-HIV patients being missed through lack of sensitivity.

### 1.8.5 Serological diagnosis of PCP – (1-3)-β-D-glucan

The detection of (1-3)-β-D-glucan is not specific for the diagnosis of PCP infection but, unlike some other fungal infections, the level of (1-3)-β-D-glucan detected during PCP infection can exceed >500pg/mL, even when IF microscopy is negative. These high concentrations of (1-3)-β-D-glucan are characteristic of PCP infection (Koga *et al.*, 2011; Damiani *et al.*, 2013). Studies have demonstrated that (1-3)-β-D-glucan negativity can be used to exclude a diagnosis of PCP with NPV > 99% as the assays show a high degree of sensitivity (Onishi *et al.*, 2012; Karageorgopoulos *et al.*, 2013). A positive result cannot be used to definitively diagnose PCP as other fungi such as *Aspergillus* to which the patient is also susceptible, contain (1-3)-β-D-glucan. Specificity of (1-3)-β-D-glucan detection ranged between 75%-86.3% in three meta analyses (Onishi *et al.*, 2012; Karageorgopoulos *et al.*, 2013; Li *et al.*, 2015). Specificity can also be impacted by false positive reactions, which have been documented for IVIG infusions (Ramsay *et al.*, 2016). The (1-3)-β-D-glucan assay has a positivity threshold >80pg/mL. If a higher threshold of >300pg/mL is used for defining a positive (1-3)-β-D-glucan result for the diagnosis of PCP it can further improve the specificity as demonstrated by Damiani *et al* (2015) reporting a sensitivity of 91% and a specificity of 92% (Damiani *et al.*, 2015). Due to the lack of specificity a positive (1-3)-β-D-glucan result should always be interpreted alongside a specific assay, such as IF or PCR, and radiological findings (White *et al.,* 2017) for the diagnosis of PCP.

### 1.8.6 PCR for the diagnosis of PCP

Recently published ECIL guidelines for PCP diagnosis, which included 34 studies of PCR testing in the report, gave the highest rating of AII (AI was not possible as randomized control trials are lacking in literature) for the use of PCR in the diagnosis of PCP (Alanio *et al*, 2016). The use of Immunofluorescence for PCP diagnosis was also

given the same AII recommendation (Alanio *et al*, 2016). A negative PCR result was given an AII rating for its ability to rule out PCP infection when testing BAL samples (Alanio *et al*, 2016). This is owing to the high negative predictive value and sensitivity of PCP PCR. Across three recent meta-analyses PCR was shown to have an NPV of ≥99% (Lu *et al.*, 2011; Fan *et al.*, 2013; Summah *et al.*, 2013; White, Backx and Barnes, 2017). The pooled sensitivity was 97-99%, demonstrating the strength of PCR in its ability to detect *P. jirovecii* in clinical specimens. As *P. jirovecii* is known to colonise the lungs, this can complicate the use of PCR as a diagnostic test for PCP (Davis *et al.*, 2008). The PPV was reported to be 66-85% and specificity 90-94%, as false positive PCR results occur when colonization is detected but the patient is not clinically diagnosed as having PCP (Lu *et al.*, 2011; Fan *et al.*, 2013; Summah *et al.*, 2013; White, Backx and Barnes, 2017. False positive PCR results (thought to be as a result of colonization) are of constant debate as a patient must be considerably sick to warrant taking a BAL for the investigation of their lung infection PCR is the only diagnostic test that can specifically detect *P. jirovecii* at low levels and it is clear that further research is required to understand the role of *P. jirovecii* when detected by PCR in cases of lung infection not thought to be PCP. This confounding issue prevents the use of PCR routinely in most diagnostic laboratories.

### 1.8.7 Prophylaxis and treatment of PCP

Due to the severity of PCP infection and the risk in HIV infected individuals and immunosuppressed patients', prophylaxis is routinely given to prevent infection. Several guidelines are available detailing criteria for selecting patients requiring prophylaxis from ECIL, the American Society of Transplantation and CDC, NIH and HIVMA/IDSA (Mofenson *et al.*, 2009; Martin and Fishman, 2013; Alexandre Alanio *et al.*, 2016). For HIV positive patients prophylaxis is recommended with a CD4 count <200 cells/μL or <14% if the patient is being routinely monitored, this increases to a threshold of 200-250 cells/μL in the absence of quarterly monitoring (Mofenson *et al.*, 2009; Martin and Fishman, 2013; Alexandre Alanio *et al.*, 2016). In haemato-oncology patients' prophylaxis should be given to acute lymphocytic leukaemia patients, allogenic HSCT patients with steroids or those receiving some forms of immune depleting therapy (e.g. Rituximab). In solid organ transplant patients all are considered at risk and requiring prophylaxis especially with graft rejection-prevention therapies, prolonged corticosteroids or neutropenia (Martin and Fishman, 2013). The length of prophylaxis is considerable, from 3-12 months across these groups and may be life-

long in some instances. A standard prophylactic regimen across all at-risk groups of oral trimethoprim/sulfamethoxazole, with dapsone or pentamidine as alternatives when trimethoprim/sulfamethoxazole is not an option therapeutically (Mofenson *et al.*, 2009; Martin and Fishman, 2013; Alexandre Alanio *et al.*, 2016). For patients diagnosed with PCP who require treatment, intravenous trimethoprim/sulfamethoxazole is recommended as empirical therapy. The alternative therapy of primaquine and clindamycin is recommended in HIV and haemato-oncology patients. For SOT patients IV pentamidine is recommended as an alternative (Mofenson *et al.*, 2009; Martin and Fishman, 2013; Alexandre Alanio *et al.*, 2016).

### 1.9 Invasive mould infection

In contrast to *Candida* and *Pneumocystis*, moulds or filamentous fungi are exogenously aquired from the environment as most fungi exist within the environment as saprophytic organisms (Carris and Little, 2012). Filamentous fungi liberate their conidia (spores) into the environment. Germination of conidia occurs when conidia enters an environment suitable to the growth requirements of that mould i.e. temperature and nutrients (Carris and Little, 2012). Once germinated a mycelial network forms and grows into a complex multicellular organism (Carris and Little, 2012). Figure 1.13 demonstrates the life cycle of a mould.

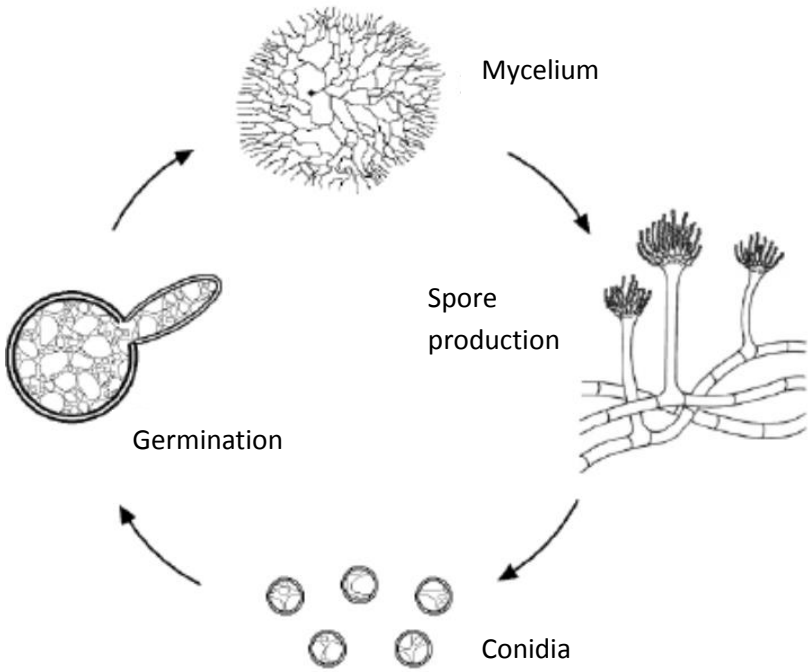



Figure 1.13 Life cycle of a mould from conidia to mycelium with fruiting structures and spore production (www.blackmould.me.uk).

Non-endemic filamentous fungi implicated in invasive mould infections are harmless to the immunocompetent population who breathe in in excess of 2000 conidia per day without any risk of infection (Boddy, 2016). For immunocompromised patients this inoculum can be deadly. Invasive Aspergillosis (IA) accounts for the main burden of invasive mould disease with greater than 95% of invasive mould infections being due to this genus (Brown *et al.*, 2012). The Prospective Antifungal Therapy registry (PATH) reported *Aspergillus* as the cause of 13.3% of all invasive fungal infections (Azie *et al.*, 2012) Non-*Aspergillus* invasive moulds infections are rare but have been reported to include fusariosis, mucormycosis and scedosporiosis (Slavin *et al.*, 2015).

### 1.10 Invasive Aspergillosis

More than a hundred species of Aspergilli have been identified to date, despite this, greater than 70% of IA results from infection by a single species, *Aspergillus fumigatus* (Ascioglu *et al.*, 2002; Kibbler, 2005; De Pauw *et al.*, 2008; Shoham and Marr, 2012). *A. fumigatus* is one of the most abundant species of fungus in the environment. Its spores are small measuring 1-2µm in size, therefore are small enough to penetrate the alveolar lung spaces when inhaled (Deacon *et al.*, 2009). *A. fumigatus* is also thermo-tolerant and is able to thrive at 37°C (Rhodes, 2006). These two factors contribute to the dominance of *A. fumigatus* as a cause of invasive fungal infection.

#### 1.10.1 Epidemiology of Invasive Aspergillosis

Patients at risk of IA include haemato-oncology patients, [especially hematopoietic stem cell transplant (HSCT) recipients], solid tumour oncology patients, solid organ transplant (SOT) patients and ICU patients (Kontoyiannis *et al.*, 2010). Recent data reported from the Transplant-Associated Infection Surveillance Network (TRANSNET) collected from 16,200 HSCT patients reported a cumulative incidence of 1.6% over a 12 month period with the highest incidence rates in the mismatched related and unrelated matched donor transplant recipients (Kontoyiannis *et al.*, 2010). In the adult intensive care setting incidence rates of 6.1 per 1000 are experienced (Tortorano, Dho, *et al.*, 2012). Unsurprisingly in the SOT patient group lung transplant recipients are most at risk of developing IA with a cumulative incidence of 2.4% compared to 0.8%, 0.3% and 0.1% for heart, liver and kidney transplants respectively (Morgan *et al.*, 2005).

Mortality rates in the haemato-oncology setting, amongst HSCT patients and in the ICU are greater than 40% (Morgan *et al.*, 2005; Nicolle *et al.*, 2011; Pacholczyk *et al.*, 2011). In the paediatric haematology/ICU setting mortality rates are much higher (Azie *et al.*, 2012). Associated risk factors include haematological malignancy, allogeneic bone marrow transplantation, immuno-suppressive therapies, granulocytopenia, corticosteroids and solid organ transplant (Fukuda *et al.*, 2003; Baddley, 2011; Kosmidis and Denning, 2015; Miceli *et al.*, 2017).

Although *A. fumigatus* causes more than 95% of cases of invasive aspergillosis, other species are rarely implicated in IA including*, A. niger*, *A. flavus* and *A. terreus* (Krishnan, Manavathu and Chandrasekar, 2009; Xavier *et al.*, 2009; Risslegger *et al.*, 2017). *Aspergillus* sp are generally susceptible to most antifungal agents but in the last decade increased levels of resistance have been reported (van der Linden *et al.*, 2015), in particular to azole antifungal agents. The high use of antifungal therapy and the agricultural use of azoles for crop control are contributing factors for the emergence of resistant strains (Vermeulen, Lagrou and Verweij, 2013; Verweij *et al.*, 2013). Recent studies have demonstrated that up to 13% of infections are caused by azole resistant strains of *Aspergillus* in the Netherlands, whereas in the UK resistance rates of 6.8 – 28% have been reported within clinical strains of *Aspergillus fumigatus* (Vermeulen, Lagrou and Verweij, 2013; Verweij *et al.*, 2013; Meis *et al.*, 2017, Howard *et al*, 2009, Mortensen *et al*, 2011).

### 1.10.2 Clinical presentations of Invasive Aspergillosis

IA is primarily a pulmonary infection resulting from inhalation of spores into the lung accounting for >90% of primary infection (Ascioglu *et al.*, 2002; Kibbler, 2005; De Pauw *et al.*, 2008; Shoham and Marr, 2012). Infection is defined by invasion of the pulmonary parenchyma by the growing hyphae of *Aspergillus* (Chabi *et al.*, 2015). Invasive Aspergillosis can be further described as being ‘angio-invasive’ demonstrating vascular invasion as described by radiological imaging (Chabi *et al.*, 2015). The clinical symptoms of IA are non-specific, including fever that is refractory to broad-spectrum antibiotics (Ascioglu *et al.*, 2002; Kibbler, 2005; De Pauw *et al.*, 2008; Shoham and Marr, 2012). Cough, chest pain and haemoptysis are documented symptoms, but not common to all infections (Ascioglu *et al.*, 2002; Kibbler, 2005; De Pauw *et al.*, 2008; Shoham and Marr, 2012). As part of the diagnostic pathway a patient who is at risk of IFI should receive a HRCT scan of the lungs to detect signs of an invasive infection (Misch and Safdar, 2016; Patterson *et al.*, 2016). Figure 1.14 demonstrates the typical

presentation of a lesion surrounded by ground glass appearance, described as the halo sign. This characteristic presentation is caused by hyphae invading new tissue inducing haemorrhage during an angio-invasive process (Georgiadou *et al*, 2011). When seen on histopathological presentation there is a strong indication for the diagnosis of Invasive Aspergillosis (Greene *et al.*, 2007; Verweij, van Die and Donnelly, 2007). However, the halo sign is transient; and has been shown to be present in 68% of patients at day 3 reducing to 19% of patients after two weeks of infection (Caillot *et al.*, 2001). IA may also present as invasive sinusitis, central nervous system infections, osteomyelitis, endophthalmitis and endocarditis resulting from dissemination or direct inoculation (Levin *et al.*, 1996; Schwartz and Thiel, 1997; Koehler, Tacke and Cornely, 2014). Symptoms of disseminated infection are related to the location of the infection with deteriorating organ function in cases of liver or renal disease. Seizures or other focal neurological signs are noted that could indicate cerebral dissemination (Kourkoumpetis *et al.*, 2012). With a lack of specific symptoms to aid diagnosis and variable radiological interpretation the diagnosis of IA also relies on clinical laboratory methodologies to detect *Aspergillus* from a variety of clinical specimens.

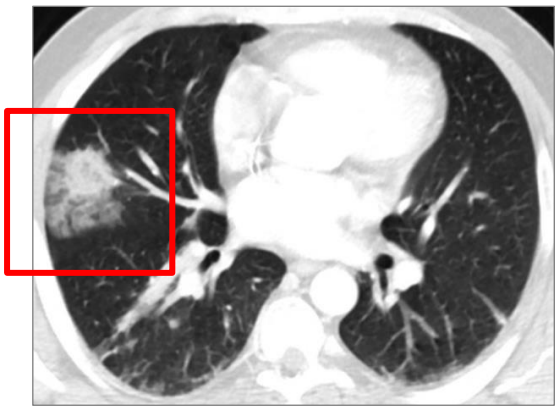


Figure 1.14 Lung lesions with the halo appearance typical of pulmonary invasive aspergillosis due to a defined circumscribed lesion surrounded by ground glass shadowing (Sheetal Shroff *et al*, 2014).

### 1.10.3 Laboratory diagnosis of Invasive aspergillosis

Conventional microbiological methods for diagnosing IA include serological based testing, specifically Galactomannan Enzyme Immune Assay (GM-EIA), direct microscopy from clinical specimens and culture (de Pauw *et al.*, 2008).

### 1.10.4 Histopathology of tissue sections

The current gold standard and most specific diagnostic method is histopathological assessment demonstrating hyphae within tissue (de Pauw *et al.*, 2008). Haematoxylin and eosin (H&E) staining will reveal the presence of *Aspergillus* hyphae whereas more specific stains such as periodic acid Schiff (PAS) and Grocott's methenamine silver (GMS) will increase sensitivity and should be carried out whenever a fungal infection is suspected (Barton, 2013). In addition to fungal hyphae the PAS staining method detects polysaccharides, glycoproteins, glycolipids and mucins in tissues (Alturkistani, Tashkandi and Mohammedsaleh, 2016). This stain is only effective with living fungi (at time of biopsy or resection) as cell walls contain high levels of carbohydrate which stain PAS-positive (Alturkistani, Tashkandi and Mohammedsaleh, 2016). In contrast the GMS stain can detect both living and dead fungi in tissues. Polysaccharides within the cell walls are oxidized to release aldehyde groups (Alturkistani, Tashkandi and Mohammedsaleh, 2016). The aldehyde groups then react with the silver nitrate, reducing it to metallic silver staining fungal elements black in colour, figure 1.16 although histopathological investigation is high in specificity in determining that a fungus is present in tissue, refining the fungal identification further is challenging. It is possible to distinguish between Ascomycetes (septate) and the Mucoromycotina (non-septate) but histopathology cannot be used to identify fungal species in most cases of infection (Vyzantiadis, Johnson and Kibbler, 2012).

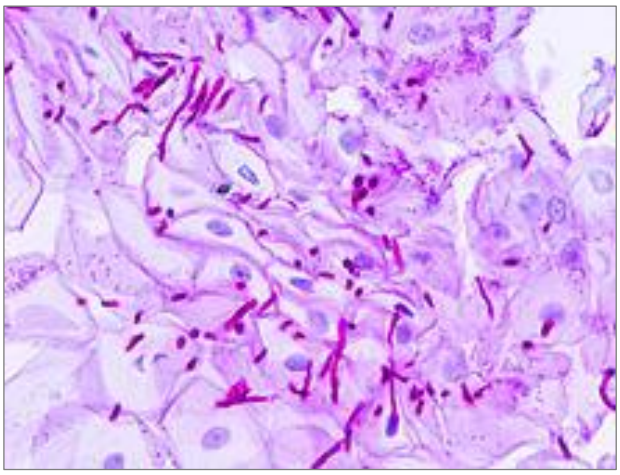


Figure 1.15 PAS stain for Invasive candidiasis (Mohammadi *et al,* 2015).

A limiting factor is that a high degree of interpretative skill is required for the accurate identification of the different fungal infections in histopathological sections. Figure 1.16 demonstrates the varying appearance of fungi in Grocott's stain from two different IFI of

Mucor and *Aspergillus*. Measuring the hyphae may assist interpretation as Aspergilli typically have hyphae with a diameter of 1-3µm (Barton, 2013). As IFI is rare, exposure to histopathological cases is limited and in centres without skilled histopathologists misidentification of fungi from sections can often occur (Vyzantiadis, Johnson and Kibbler, 2012). For this reason reports are often limited to stating 'hyphae' or 'filamentous fungi seen'.

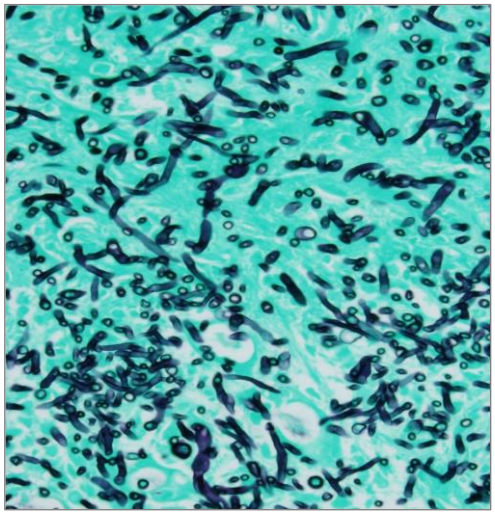
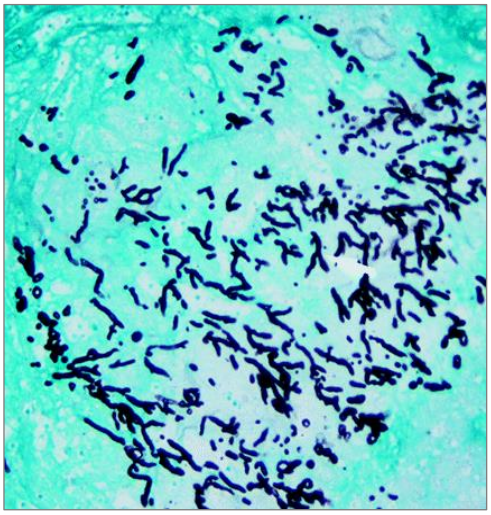
**a)**  **b)** 

Figure 1.16 Grocott's staining for fungi in histopathological sections of tissue, from left to right showing Mucor sp and *Aspergillus* fumigatus (Cho *et al.*, 2007; Chow *et al.*, 2015).

### 1.10.5 Microscopy and culture of *Aspergillus* sp from clinical specimens

If a fresh biopsy is available specimen culture is the conventional approach for the recovery of *Aspergillus*. Other specimen types such as sputum, BAL aspirates and sterile fluids such as CSF may also be sent for investigation.

Direct microscopy should always be performed on sterile specimens and BAL (Schelenz *et al.*, 2009, 2015). Direct microscopy can also be performed on sputum, although this is not routinely practiced, as the analysis of sputum using fluorescent microscopy may lead to false positive results. Commonly a fluorescent brightener, calcofluor, is added to the specimen on a slide and analysed using fluorescence microscopy (Harrington and Hageage, 2003). Direct microscopy lacks specificity as the fruiting structures required for identification of *Aspergillus* rarely occur *in vivo.* Specimen quality also can have an impact on the results (Kozel and Wickes, 2014). If hyphae are seen they may be from any number of filamentous fungi. Dichotomous

branching has been used to try and distinguish *Aspergillus* from other mould species as in figure 1.16 b. The accuracy of histology for the identification of fungi when compared with culture was reported to be 83% (Kozel and Wickes, 2014).

Culture for *Aspergillus* in suspected infection should always be undertaken. Isolating *Aspergillus* from infection is the only clinical investigation that enables vital susceptibility testing to be performed, guiding targeted antifungal therapy. *Aspergillus* sp are relatively rapid in growth (typically will grow within 48 hr) and will culture on most microbiology media including selective Sabouraud's agar but also on standard bacterial agar such as blood agar. However, the sensitivity of culture is less than optimal. Table 1.6 details culture rates based on specimen type, overall sensitivity does not exceed 62%, and from sputum can be as low as 8% (Denning, 2005).

**Table 1.6 Sensitivity of culture for *Aspergillus* from clinical specimens (Denning, 2005)**

| Specimen | Culture rate |
|---|---|
| Sputum | 8 – 34% |
| BAL | 45 – 62 % |
| Blood/CSF | Rarely positive |
| Tissue | 56% |

The sensitivity of culture for *Aspergillus* is most likely affected by a lack of standardised laboratory sample preparation methods, with standards for microbiological investigations (PHE Surveillance Services, 2015) unsuitable for fungal workup. The current SMI of sputum in the U.K recommends a 1µL loop of sputum to be inoculated onto a Sabouraud's agar slope. Fungal elements within sputum are not as abundant as bacteria so it is likely that a much larger volume of sputum is required for optimal fungal recovery. A recent study by Fraczek *et al* (2014) improved sensitivity of culture from respiratory specimens was reported by using higher volume cultures, with 44% of sputum samples positive for *Aspergillus* compared with 0% using the SMI methodology. Higher volume cultures resulted in the x100 fold increase in the number of fungal colonies isolated from clinical specimens. This alone suggests the culture of *Aspergillus* from clinical specimens requires significant improvement and standardisation to enhance its reproducibility, clinical accuracy and ultimately its usefulness in diagnosing IA (Fraczek *et al.*, 2014).

If *Aspergillus* is isolated from clinical specimens the interpretation and identification of most *Aspergillus* species is straightforward (de Hoog and Guarro, 1995). Macroscopic and microscopic characteristics, including colony forming morphology, growth rate and

morphology of fruiting structures such as the vesicles are used to accurately determine identification. Occasionally atypical forms of *A. fumigatus* may be encountered, particularly in patients who have been exposed to mould active antifungal agents (Brandt *et al*., 2009). However, the epidemiology of IA is changing as molecular methods have led to the description of infections with de novo species *A. lentulus*, *A. novofumigatus,* and *A. fumigatiaffinis*. These species cannot be distinguished morphologically from *A. fumigatus* and require DNA based sequence analysis to reliably confirm their identification (Peláez *et al.*, 2013; Ola *et al.*, 2014; de Azevedo Bastos *et al.*, 2015). Most diagnostic laboratories do not routinely sequence their *Aspergillus* isolates therefore species remain undetected until a method with higher resolution in identification that is applicable in the routine diagnostic setting is developed.

### 1.10.6 Serological diagnosis of Aspergillosis

The cell walls of *Aspergillus* spp. contain the carbohydrate molecule Galactomannan (GM), which is released from the cell wall during fungal growth (Morelle *et al.*, 2005). During invasive infection the release of GM has been correlated with *Aspergillus* invasion of the endothelial compartment (Hope *et al.*, 2007). Therefore, GM detection should be a strong indicator of angioinvasion. The Platelia *Aspergillus* galactomannan antigen sandwich enzyme immunoassay (GM-EIA) (Bio-Rad, California, USA) is used globally as a screening method for prospective surveillance of invasive aspergillosis (IA), usually through twice weekly testing of serum samples, in patients at high-risk of disease. BAL specimens can also be processed. The GM-EIA assay is now well established, reflected by its recommendation in European Organisation for Research and Treatment of Cancer (EORTC) MSG consensus criteria for defining invasive fungal disease (de Pauw *et al.*, 2008).

Despite this the diagnostic performance of the GM-EIA is variable, with meta-analyses showing combined sensitivity and specificity ranges of 71%-78% and 81%-89% respectively (Pfeiffer, Fine and Safdar, 2006; Leeflang *et al.*, 2008). False positivity experienced using the GM-EIA has been associated with antimicrobial preparations involving Penicillium (e.g. piperacillin-tazobactam) as well as ingestion of ice pops and when testing patients with histoplasmosis infection (Pfeiffer, Fine and Safdar, 2006; Gerlinger *et al.*, 2012; Tortorano, Esposto, *et al.*, 2012; Guigue, Menotti and Ribaud, 2013). The assessment of compounds classified as interfering substances is part of the In Vitro Diagnostic (IVD) regulations to bring a commercial assay to market. These

substances should be tested as part of the assay validation and findings reported to users within the assay interpretation guidelines. Laboratories are required to work directly with clinical teams to ensure where possible interfering substances are limited.

Although initially, the GM-EIA assay reproducibility was reported to have high inter-laboratory reproducibility (Pedroza *et al.*, 2013), recent reports document a lack of precision when repeat testing positive samples (Furfaro *et al.*, 2012). In particular, samples with an optical density index (ODI) value on or around the positivity threshold of the assay (0.5) are regularly found to be negative on repeat testing (Upton *et al.*, 2005; Kimpton, White and Barnes, 2014). The storage conditions of the primary specimen appears to impact on reproducibility with a significant decline in sample ODI values reported after storage at -80°C (Kimpton, White and Barnes, 2014). IA diagnosis also appears to be important, with a lack of reproducibility more frequently observed when retesting false positive samples from patients without IA (Furfaro *et al.*, 2012; Kimpton, White and Barnes, 2014). A significant correlation between serum albumin concentration and difference in ODI value on retesting has also recently been reported (Upton *et al.*, 2005). Improved standardisation of the GM EIA has been demonstrated by automating the assay on an ELISA processing platform (Gorton *et al.*, 2015). However, due to the respective performance limitations the GM-EIA cannot be used as a stand-alone diagnostic test but is an important component of the diagnostic testing strategy for IA.

### 1.10.7 PCR for the diagnosis of Invasive Aspergillosis

The use of PCR for the diagnosis of invasive aspergillosis remains a debated issue despite recent advances in standardisation, primarily through the work of the European *Aspergillus* PCR Initiative (EAPCRI) (White *et al.*, 2010). Performance of PCR is heavily dependent on the specimen type being investigated. The main two specimens used for the detection of *Aspergillus* from patients suspected of having IA are BAL and blood. PCR testing on BAL was first published in 1993 with multiple studies showing consistent performance with sensitivity and specificity rates ranging between 76.8-79.7% and 93.7-94.5% respectively (Tuon, 2007; Sun *et al.*, 2011; Avni *et al.*, 2012). PCR from BAL is clinically useful for ruling out infection with a high NPV. When considering PCR from blood specimens these must be broken down into whole blood, serum and plasma. The performance of PCR from the different fractions of blood varies. When comparing whole blood to serum the sensitivity is 85% compared with 79% respectively, but the specificity is lower for whole blood 65% compared with 84%

for serum (Springer *et al.*, 2016). The investigation of plasma as an alternative to serum shows superior sensitivity 95% compared with 68% for serum (White *et al.*, 2015). As serum or plasma can be processed using simplified extraction methods, as opposed to whole blood that can be complicated due to the requirement of centrifugation, red cell lysis and washing stages, serum and plasma present as the more optimal sample types for *Aspergillus* PCR. In a recent meta-analysis Mengoli *et al* reported that a single positive PCR reaction alone was not sufficient for the diagnosis of IA as the sensitivity and specificity were reported as 0.88 (95% CI 0.75-0.94) and 0.75 (95% CI 0.63-0.84), respectively (Mengoli *et al.*, 2009). *Aspergillus* PCR is not used routinely by most laboratories for this reason and is confined to use in specialist mycology laboratories.

### 1.10.8 Prophylaxis and treatment of invasive aspergillosis

The use of prophylaxis for patients at risk of developing IA is in part due to the aforementioned lack of robust diagnostic testing pathways to rapidly detect an infection. Currently empirical treatment of invasive aspergillosis as the first line recommendation is IV voriconazole, table 1.7. Isavuconazole, a recently released broad spectrum azole, was also given the highest rating by the ECIL-6 (2016) and IDSA guidelines (2016) as the first line therapy (Thomas F Patterson *et al.*, 2016; Tissot *et al.*, 2016). An alternative first line option is liposomal Amphotericin B but only in patients unable to receive azoles or in the rare instance of knowing an isolate is resistant to azole antifungals.

**Table 1.7 ECIL and IDSA recommendations for the management of invasive Aspergillosis (Thomas F Patterson *et al.*, 2016; Tissot *et al.*, 2016)**

| | ECIL-6 | IDSA |
|---|---|---|
| First line treatment | voriconazole (AI*) | voriconazole (strong high quality*) |
| Alternative | Isavuconazole (AI*) | Isavuconazole (strong moderate*) |
| Alternative | Liposomal Amphotericin B (BI*) | Liposomal Amphotericin B (strong moderate*) |

*see table 1.2 for overview of evidence ratings

The main stay of prophylaxis, as recommended by 2008 consensus antifungal guidelines is Itraconazole or posaconazole (Slavin *et al*., 2008). Both antifungals can be administered orally providing an advantage in the management of this patient group.

### 1.11 Non-*Aspergillus* invasive mould infection

There are many species of fungi reported in the literature as etiological agents of IFI. Immuno-compromised patients are susceptible to any mould that presents a risk of infection. Some fungi are more commonly encountered than others in IFI. The incidence of non-*Aspergillus* invasive mould infection is difficult to define, as the incidence is very low in comparison to other invasive fungal diseases. Two of the most common infections are mucoraceous mould infections, with an estimated annual incidence of >10,000 cases globally (Brown *et al.*, 2012), and fusariosis for which an estimated global incidence has not been reported. In the high-risk haemato-oncology setting fusariosis cases range from 0.8-3.1 per 1000 patients (Garnica *et al.*, 2015) and are responsible for 3-5% of all invasive fungal disease (Rostaing and Malvezzi, 2016). The majority of non-*Aspergillus* fungi are found in the environment either within compost heaps and decaying vegetation (Mucoraceous mould) (CDC, 2011) or are wet moulds found within sewers or ponds/lakes (*Scedosporium*/*Fusarium*) or other environments of this type (Luplertlop *et al.*, 2016).

#### 1.11.1 Epidemiology of non-*Aspergillus* invasive mould infection

A small number of studies have been published on the epidemiology of non-*Aspergillus* IFI. The largest study by Slavin *et al (*2015) details infections reported across a six-year time span in Australia (Slavin *et al.,* 2015). Over 23 fungal species were reported from 180 proven/probable invasive fungal infections (Slavin *et al.*, 2015). The most common fungi included those of the Mucoromycotina, *Scedosporium* sp and *Fusarium* sp. In two similar studies published on data generated from the USA a similar distribution of fungal species was also reported (Park *et al.*, 2011), table 1.8. The respective species are the commonest non-*Aspergillus* species implicated in IFI and will be considered as a representation of the diverse group of non-*Aspergillus* invasive fungal infections.

In a recent report by Guinea *et al* there was a significant increase in the incidence of mucormycosis from 1.2 cases/100,000 hospital admissions between 1988-2006 to 3.3 cases/100,000 hospital admissions between 2007-2015 (Guinea *et al.*, 2017)

Mucormycosis accounts for the greatest proportion of non-*Aspergillus* mould infections in the USA and Australia (Slavin *et al.*, 2015) and globally (Lass-Flörl and Cuenca-Estrella, 2017). In the adult population mortality rates associated with non-*Aspergillus* mould infection can range from 20-50% (Lass-Flörl and Cuenca-Estrella, 2017). The most commonly reported risk factor for Mucormycosis being uncontrolled diabetes mellitus (Vijayabala *et al*., 2013). Other risk factors for mucormycosis IFI include haematological disorders, solid organ transplant and trauma patients (Wu, Zhang and Jiang, 2010; Petrikkos *et al.*, 2012; Lelievre *et al.*, 2014; Mitchell *et al.*, 2014; Kermani *et al.*, 2016; Moreira *et al.*, 2016; Zahoor, Kent and Wall, 2016) amongst other immunosuppressive conditions. The most commonly implicated fungi include *Mucor sp*, *Rhizopus sp* and *Lichthemia sp* accounting for 70-80% of mucormycosis (Wu, Zhang and Jiang., 2010; Petrikkos *et al*., 2012; Leiievre *et al*, 2014; Mitchell *et al*., 2014; Kermani *et al*,. 2016; Moreira *et al*., 2016; Zahoor, Kent and Wall., 2016).

**Table 1.8. Documented proven and probable cases of non-*Aspergillus* infections from two large multi-center surveillance studies in Australia and the USA.**

| Organism | **2004-2012** (Slavin *et al.*, 2015) | **2001-2006** (Park *et al.*, 2011) |
|---|---|---|
| | Australia | USA |
| Mucoromycotina | 74 | 105 |
| *Scedosporium* sp | 54 | 27 |
| *Fusarium* spp | 13 | 37 |

Fusariosis is more frequently described as an emerging fungal infection with increasing incidence (Nucci *et al.*, 2015) but infection remains rare. Fusariosis is associated with 48-90% mortality (Lass-Flörl and Cuenca-Estrella, 2017) but can be lower with 44% survival as reported from a review of 65 cases (Horn *et al.*, 2014). Patients at risk of fusariosis are the same at-risk patient group as for IA, including neutropenia, corticosteroids, HIV, haematological malignancies, transplant and patients with prosthetic devices (Dabas, Bakhshi and Xess, 2016). As this genus of moulds are commonly associated with wet environments further risk factors include working in or exposure to contaminated wet environments (Peman and Salavert, 2014). Over 300 *Fusarium* species have been described with the most frequently implicated species in infection being *F. solani*, *F. oxysporum* and *F. moniliforme* (Horn *et al.*, 2014). The most important factor clinically when treating Fusariosis is the choice of antifungal therapy. There is a wide range of variability across species with regards to

susceptibility to antifungal agents, presenting a challenge for the selection in clinical practice. Identifying the *Fusarium* species causing an infection is crucial for optimal management of infection (Muhammed *et al*., 2011)

*Scedosporium* sp, like *Fusarium*, are wet moulds found in sewage, soil, water and other areas mostly with decaying vegetation. Acquisition of infection is either through contact with contaminated environments or inoculation of inhalation/ingestion of the fungus (Tadros *et al.*, 1998; Nesky, McDougal and Peacock Jr, 2000). Primary sources of infection are the lungs and open wounds. Patients at risk of being infected by *Scedosporium* are the same patients as *Fusarium* and *Aspergillus*. In a review by Johnson *et al* (2014) of 57 cases in solid organ transplant patients the mortality rate was 54%. Scedosporiosis is generally caused by two species, *S. apiospermum* and *S. prolificans* (Johnson, Shields and Clancy, 2014). *S. apiospermum* reportedly causes a greater proportion of infections than *S. prolificans* (Johnson, Shields and Clancy, 2014). Both species are intrinsically resistant to Amphotericin B (Ambisome®) but infection with *S. prolificans* is complicated as this species conveys greater intrinsic resistance to most antifungal agents (Wang *et al.*, 2015; Goldman *et al.*, 2016). Choice of effective therapy is again reliant on accurate diagnosis of infection through speciation of the fungus. A wide range of infections have been reported including bone and joint infections and also haematogenous spread and dissemination (Peman and Salavert, 2014).

### 1.11.2 Clinical presentation of Non-*Aspergillus* invasive mould infection

Spores of mucoraceous moulds are large in comparison to other fungi, measuring 7-10μm in diameter compared with *Aspergillus* conidia measuring 1-3μm in diameter (Wang *et al*., 2018). Infection presents commonly as rhino-cerebral disease, as the spores are lodged inside the nasal cavity. Pulmonary infection can occur and has a higher incidence in haemato-oncology patients. Wound infection post trauma is a recognised complication in scenarios such as road traffic accidents, military personal and in the aftermath of natural disasters such as tornado (Wu, Zhang and Jiang, 2010; Binder, Maurer and Lass-Flörl, 2014; Riley *et al*., 2016). Infections with mucoraceous fungi are aggressive and tissue damage is characterised by severe necrosis, becoming black as the infection progresses (Wu, Zhang and Jiang, 2010; Binder, Maurer and Lass-Flörl, 2014; Riley *et al*., 2016). Patients present with fever and sore sinus and within hours this will have progressed to proptosis and cerebral pain, blurred vision and a breakdown in the facial tissue. In pulmonary or wound infection disseminated disease

via haematogenous spread is more likely to develop; with characteristic blackened skin lesions often present at the source of infection (Wu, Zhang and Jiang, 2010; Binder, Maurer and Lass-Flörl, 2014; Riley *et al*., 2016).

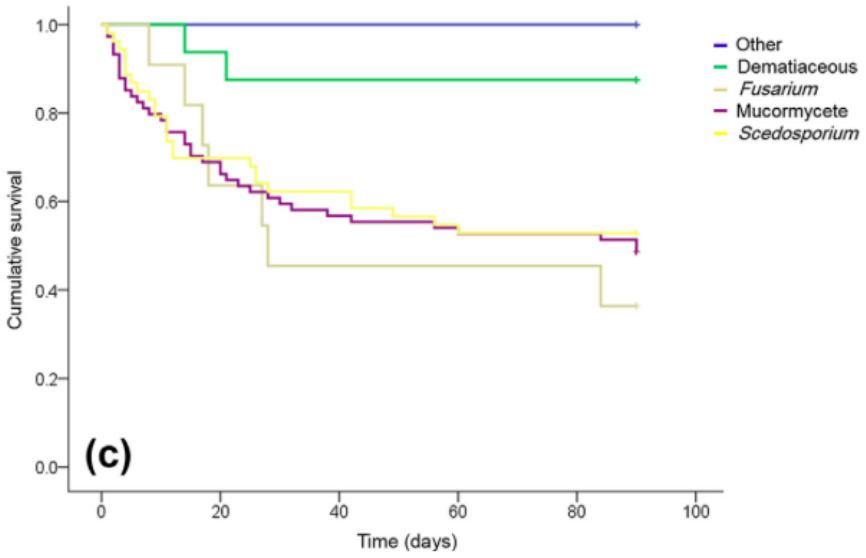



Figure 1.17. Survival curve for non-*Aspergillus* fungal infection from 180 cases recorded over 6 years in Australia (Slavin *et al*., 2015).

Fusariosis and scedosporiosis presents typically as a pulmonary infection (cough and dyspnoea), with a sepsis like syndrome and fever refractory to antimicrobials (Husain *et al*., 2005; Cortez *et al*., 2008; Peman and Salavert, 2014). Hypotension has been noted in HCST patients (Husain *et al*., 2005; Cortez *et al*., 2008; Peman and Salavert, 2014). Haematogenous spread is more likely in HCST patients, and was noted in 33% of HSCT patient compared with 11% in SOT patients for Scedopsorium infection (Hussein, rodriguez-tudela). In disseminated infection, skin lesions are commonly reported with 36% of HSCT recipients reported to have cutaneous involvement with scedosporiosis (Husain *et al*., 2005). Infection through inoculation is commonly reported in patients with a wound at the site of infection. The infecting species can impact on the clinical presentation, with *S. prolificans* being significantly more associated with fungaemia compared with *S. apiospermum* infection (P = .009) (Husain *et al*., 2005). Central nervous system involvement is common and patients will present with sign of cerebral infection (Rodriguez-Tudela *et al*., 2015). In figure 1.17 the survival rates of patients with non-*Aspergillus* invasive infection are plotted over days. Mucoraceous infection, scedosporiosis and fusariosis have similar survival rates with 40% mortality at 20 days reducing to 45-50% mortality after day 50 (Slavin *et al*, 2015).

### 1.11.3 Laboratory diagnosis of Non-*Aspergillus* invasive mould infection

### 1.11.4 Microscopy for the detection of non-*Aspergillus* IFI

Direct miscopy on clinical specimens can be performed using a fluorescent brightener such as Calcofluor on tissue, fluid and respiratory specimens. This test is usually performed if there is a high degree of suspicion for fungal infection. Performance data for direct microscopy is lacking in the literature. There is a single key difference in the appearance of mucoraceous fungi compared to ascomycete fungi in direct microscopy preparations, that being the size of the hyphae (Hageage and Harrington, 1984). Figure 1.18 illustrates images of mucoraceous fungi stained with calcofluor compared with *Aspergillus*. Hyphae of mucoraceous fungi are wide ribbon like hyphae, septa are infrequent and the hyphae appear irregular. Ascomycetes have thinner hyphae, septa are clearly visible and branching is defined with regular lines of the hyphae (Hageage and Harrington, 1984). If a mucoraceous infection is suspected the ability to distinguish the presence of hyphae direct from specimen is crucial to rapid diagnosis (Hageage and Harrington, 1984).

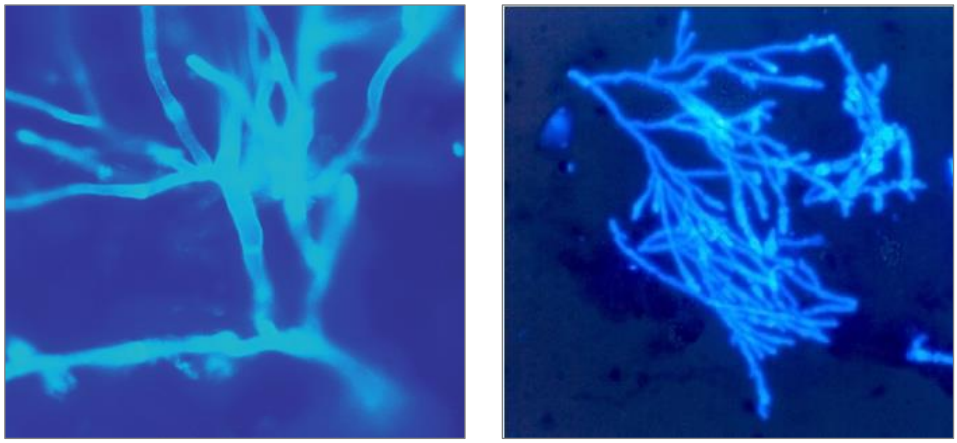


Figure 1.18 Calcafluor staining of a mucoraceous mould versus an ascomycete mould (x40 objective) (Kalkanci and Ozdek, 2011; Vanzzini Zago, Alcantara Castro and Naranjo Tackman, 2012).

### 1.11.5 Fungal culture

The performance of fungal culture for the diagnosis of non-*Aspergillus* fungal infections is a standard of care within diagnostic microbiology laboratories (Barnes *et al*., 1996; Schelenz *et al*., 2015). Specimens are inoculated onto Sabouraud's agar, either on

plates or slopes and cultured typically at 30°C for up to 3 weeks. In general, cultures are insensitive, but data are lacking on the performance characteristics for the isolation of non-*Aspergillus* species. For mucoraceous infection it is estimated that <25% of sputum specimens will be positive by culture for these fungi (Denning, 2005). As cultures typically grow within 24 hours a rapid identification can be achieved if cultures are positive (Denning, 2005). There is also some utility in the use of blood cultures for the isolation of *Fusarium*, with 40-60% sensitivity reported (Denning, 2005). All cultures from specimens with a suspicion of invasive fungal infection should be treated as significant and where possible full species identification achieved to enable the correct antifungal therapy to be administered. Identification is performed using microscopy and stains such as lactophenol cotton blue (Campbell, 2013). Macro- and micro-morphological characteristics are utilised for species identification. The accuracy of identification for some fungal species is currently sub-optimal, especially for *Fusarium* species. This is reflected in recent studies reporting the accuracy of conventional identification by microscopy to be between 61.5%-80.1% (Becker *et al*., 2014; Gautier *et al*., 2014; Ranque *et al*., 2014). For non-*Aspergillus* isolates successful identification to the species level can be as low as 31% in the laboratory when compared with gold standard molecular identification (Ranque *et al*., 2014). Due to the inaccuracies in identification of fungi from culture using microscopy, laboratories often turn to molecular methods for accurate identification of the fungal species.

**Table 1.9 Sensitivity of culture for non-*Aspergillus* IFI**
(Denning, 2005)

| Genus | Specimen | Positivity rate |
|---|---|---|
| *Fusarium sp* | Blood | 40 - 60% |
| *Mucor sp* | Sputum | <25% |

### 1.11.6 PCR for the detection and identification of non-*Aspergillus* fungi

The use of PCR for the identification of fungal isolates or fungi direct from clinical specimens is not routinely performed in most laboratories. In general this remains a function of highly specialised laboratories and reference facilities. Pan-fungal PCR provides an opportunity to test specimens where fungal culture is negative but hyphae have been observed microscopically, or to identity fungal colonies more accurately.

There are many studies investigating the use of pan-fungal PCR, with 80-100% concordance with histopathology reported direct from tissue (Paterson *et al*., 2003; Bialek, 2005; Coura, 2005). Real-time PCR has been used to identify fungi from tissue biopsies with a sensitivity of 100% (Springer, Lackner, *et al*., 2016). The choice of target is important as 18S ribosomal PCR assays are specific to the genus level only, whereas, assays that target the intergenic spacer region (ITS) of the ribosomal genes are more specific and can identify fungi to the species level (Borman *et al*., 2008). The identification of *Fusarium* species can be more complicated and a combination of targets are usually utilised (Geiser *et al*., 2004). The use of pan-fungal PCR to identify fungi can take up to 3 days for a result as amplicons require sanger sequencing, however faster turnaround times have been demonstrated using pyrosequencing techniques. Both modes of sequencing come with additional cost in terms of laboratory labor and time therefore; the use of these methods for the identification of fungi should be clinically justified with a degree of control. There is a need of a more accurate, rapid and routine method for the identification of non-*Aspergillus* moulds isolated from clinical specimens.

### 1.11.7 Antifungal therapy and management of non-*Aspergillus* invasive fungal infection

The optimal treatment for non-*Aspergillus* fungal infection has yet to be defined, as there are wide variations in the antifungal susceptibility of non-*Aspergillus* fungi. For mucoraceous fungal infection surgical debridement is an essential intervention, in an attempt to remove as much of the infected tissue to prevent spread of the infection (Cornely, Arikan-Akdagli, *et al*., 2014; Tissot *et al*., 2016). As infections are often rhino-cerebral in presentation this treatment is not always possible. Antifungal therapy is usually IV high dose liposomal amphotericin B (Ambisome®). posaconazole (IV) has shown some potential as an alternative agent. In reality when faced with mucoraceous fungal infection combination therapy is often administered. The treatment of scedosporiosis and fusariosis is heavily dependent on the causative species and in the absence of identification voriconazole if often administered as first line antifungal therapy.

### 1.12 Molecular diagnostic tool box

There are a variety of molecular techniques that can be applied to the diagnosis of infectious diseases. Strengths and limitations of these techniques should be considered when selecting which method is best suited to the disease being diagnosed. In essence a molecular toolbox is available to scientists that can be used to improve the detection of microbial pathogens from clinical specimens.

#### 1.12.1 Parameters used to determine the performance of a diagnostic test

When developing or selecting a method for use as a diagnostic test several parameters need to be considered; a) the assessment of the method's ability to provide an accurate diagnostic result, b) the reproducibility and complexity of the test and c) cost. Diagnostic accuracy is measured against a gold standard.

1. **Gold standard**; the gold standard can be a test (or a combination of tests) that is considered the current preferred method of diagnosing a particular disease either offered within an existing service or performed elsewhere (e.g. in the context of centres of clinical excellence or specialist referral laboratories). Alternatively clinical classification of patients according to defined and agreed criteria can also be utilised as the gold standard, this approach is typically used for measuring performance of fungal diagnostics as there is a lack of a series of gold standard analytical tests for comparison (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

| | | **Presence of disease (Gold standard)** | | | |
|---|---|---|---|---|---|
| | | **Positive** | **Negative** | | |
| **Test outcome** | **Positive** | True positive (A) | False Positive (B) | **PPV** | A/A+B |
| | **Negative** | False negative (C) | True negative (D) | **NPV** | C/C+D |
| | | **Sensitivity** | **Specificity** | | |
| | | A/A+C | D/B+D | | |

Figure 1.19 four by four table for the calculation of test performance parameters against a gold standard, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

2. **Analytical Sensitivity and Limit of detection (LOD);** the sensitivity of a test is its ability to correctly detect a disease. To calculate sensitivity you must divide the true positive result by the true positive plus the false negative results (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

3. **Specificity:** is a measure of a test's ability to correctly determine that control patients as not having disease. To calculate specificity you must divide the true negatives by the true negatives plus the false positives (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

4. **Positive predictive value;** is essentially a percentage of the patients with a positive test who have the disease. To calculate the PPV you must divide true positive result by the true positive plus the false positive results (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

5. **Negative predictive value;** is the ability of the test to determine the percentage of patients who do not have the disease. This value is obtained by dividing true negatives by false negatives plus true negatives (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

6. **Accuracy and precision;** the accuracy of a test is its ability to reproduce the same result repeatedly without variation irrespective of the value. Precision relates to the ability of a test to obtain the expected result. In figure 1.20 accuracy and precision are represented in bull's eye diagrams, demonstrating how accuracy can be high but the result could be imprecise (Altman and Bland, 1994; Lalkhen and McCluskey, 2008).

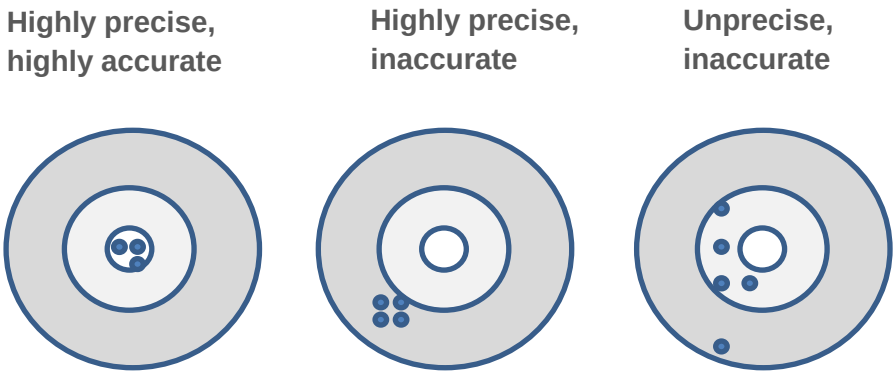



Figure 1.20 Diagrammatic representations of precision and accuracy.

### 1.12.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was first referenced in 1985 (Strauss, 1985) and over the last 30 years has developed exponentially in utility. The implementation of thermostable polymerases and advances in thermal cycler technology have allowed PCR to embed as a routine technique used throughout diagnostic laboratories. This technique supports a myriad of clinical applications in areas such as infection, acquired and hereditary malignancies cancer and pediatric and adult genetic disorders (Kaltenboeck and Wang, 2005). Despite considerable development in PCR technology, both in terms of instrumentation and chemistry, the basic principles to date still include formulation of a master mix consisting of primers, buffer (e.g. $MgCl_2$), nucleotides and DNA polymerase to which the DNA template is added. PCR occurs through reaction cycles (35-50) consisting of DNA denaturation (95°C), primer annealing (60°C) and extension of the amplicons (72°C) eventually resulting in the exponential amplification of the target. Two PCR methods are routinely used within laboratories, conventional and real-time PCR (Kolmodin and Williams, 1997; Raeymaekers and Raeymaekers, 2000; Gualberto, 2004). The latter was first developed in the late 1990's. The fundamental difference between the two methods is the process of detecting amplification. Conventional PCR uses end point detection through visualising amplicons in solid phase, most commonly by gel electrophoresis, see figure 1.21 a) (Gualberto, 2004). The reaction mix is transferred into an agarose gel containing reagents that enable the amplicon to fluoresce, such as ethidium bromide. An electrical current is passed through the gel and the amplicons migrate according to their size. Detection occurs by placing the gel into an imaging platform and ultra violet (UV) light is passed through the gel whilst a camera images the result, figure 1.21 a) (Gualberto, 2004). This method of detection adds an additional 1-2 hours onto the turnaround time for a PCR result.

In contrast real-time PCR amplification does not require any post PCR product manipulation as the amplification is visualised in real time as a measure of fluorescence emitted from labeled probes complementary to the target being amplified (Raeymaekers and Raeymaekers, 2000; Arya *et al*., 2005). As the amount of amplified target increases more probe binds to its target in real time and the fluorescent signal increases, figure 1.21 b).

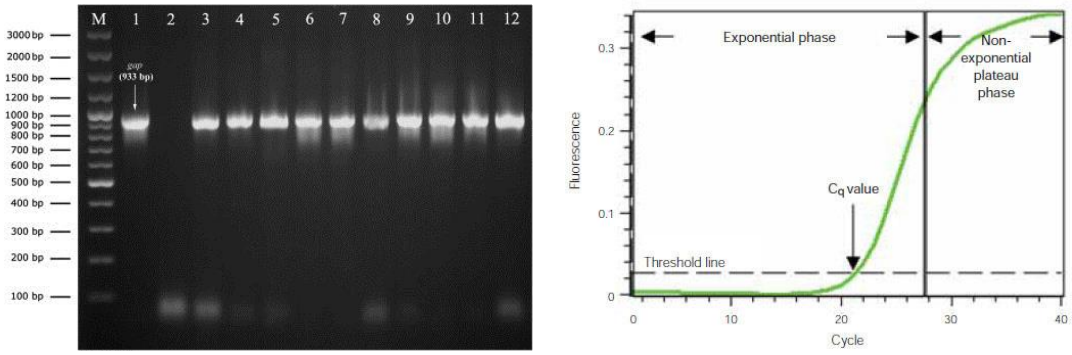



Figure 1.21. a) Detection of amplification for conventional PCR (gel electrophoresis) and b) real-time PCR (fluorescence detection in real time).

Both methods are still utilised in routine diagnostics. However, real time PCR has become the method of choice in most diagnostic laboratories, in part because of the ability to also quantify target within a specimen (Raeymaekers and Raeymaekers, 2000; Arya *et al*., 2005). Real time PCR amplification allows a positivity threshold to be assigned which defines the detection point of amplification. As fluorescence increases and crosses the threshold a quantification cycle, C*q*, is assigned to the result [figure 1.21 b)] (Raeymaekers and Raeymaekers, 2000; Arya *et al*., 2005). The C*q* value relates directly to the amount of target present within the sample. It is generally accepted that a change in C*q* of 3 cycles is equal to a ten-fold change in concentration of the target (Raeymaekers and Raeymaekers, 2000; Arya *et al*., 2005). By generating a calibration curve, determined from known concentrations of target, the number of copies of the target within the specimen can be calculated. Therefore real-time PCR can be qualitative or quantitative dependent on the format results are reported in, whereas conventional PCR in general is qualitative only.

One of the main strengths of PCR as a molecular diagnostic tool is very high sensitivity. Sensitivity of PCR is directly related to limit of detection (LOD) of the assay. The LOD of PCR is determined through analysis of serial dilutions of biological replicates and the higher dilution that achieves 95% positivity is deemed to be the LOD (Raeymaekers and Raeymaekers, 2000; Gualberto, 2004; Bustin, A *et al*., 2009). In theoretical analyses the LOD of PCR was reported to be equal to or greater than three copies of the nucleic acid targets. However, studies have demonstrated PCR assays such as those using digital droplet PCR methodologies that are capable of detecting a single copy of target DNA molecule from a variety of specimens (Hassan *et al*., 2006). The high sensitivity of PCR is made possible through specificity in the design of the

assay primers complementary to the target gene sequence of known pathogens. Loci for taxonomic identification (such as multi-copy ribosomal coding regions) are generally used for detection of pathogens but other targets can include phenotypic traits (such as antimicrobial resistance determinants), or virulence factors (Mackay, 2004; Mothershed and Whitney, 2006). Primer sequences are short oligonucleotide sequences designed to complement target genes encoding species-specific targets. The binding of primers to the target sequence enables the polymerase recognition and subsequent replication in the 5'-3' direction (Gualberto, 2004). The choice of target determines the specificity of the PCR both in its ability to be unique to the species of the pathogen being investigated but also inclusive enough to detect all strains of that species. Primer design has to satisfy several parameters including; primer length (optimally 18-22 nucleotides), a melting temperature between 52-58°C, similar annealing temperatures, GC content between 40-60% and avoidance of secondary structure formation whereby the primer loops back and sticks to itself (Gualberto, 2004). Several software packages are available now that can analyse a target sequence and generate primer sequences. In support of development of in-house or commercially available assays these include but are not limited to Primerdesign, Primer BLAST, Primer3. It is a requirement of commercial assays that the manufacturer should demonstrate specificity as part of the assay validation, which must be verified by the end user before clinical use in the diagnostic laboratory (ISO:15189., 2012).

The highly sensitive and highly specific features of PCR have transformed the diagnosis of infectious diseases. The first field in the infection sciences to develop services utilising PCR was virology (Mackay, Arden and Nitsche, 2002; Gunson, Collins and Carman, 2006; Gunson *et al*., 2008). Viruses were difficult to detect, requiring culture in cell lines, such as monkey kidney cells, a process which is extremely time consuming and technically impossible in some instances (Mackay, Arden and Nitsche, 2002; Gunson, Collins and Carman, 2006; Gunson *et al*., 2008). Diagnosis of viral infection relies heavily on serological based testing for the detection of the host response to viruses through antibody or viral antigen detection, this process is still employed as front line screening but due to sensitivity and specificity issues antigen and antibody testing has been replaced in some instances by PCR based methods (Mackay, Arden and Nitsche, 2002; Gunson, Collins and Carman, 2006; Gunson *et al*., 2008). Molecular methods have since been introduced as confirmatory and in some cases front line detection methods. The implementation of PCR for the amplification of RNA and DNA viral targets advanced diagnostic virology into what is now a leading edge PCR based diagnostic service (Mackay, Arden and Nitsche, 2002;

Gunson, Collins and Carman, 2006; Gunson *et al*., 2008). In some circumstances established PCR and sequencing based methods are now being replaced by whole genome sequencing using next generation technology (Parikh *et al*., 2017). Bacteriology is now following the footsteps of virology with PCR based detection being the method of choice for hard-to-culture microorganisms. Atypical pneumonia caused by *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophila* relied heavily on serological methods to diagnose infection but increasingly PCR is being used to detect the atypical bacteria from respiratory specimens (Oosterheert *et al*., 2005; Ling and McHugh, 2013). The detection of *Mycobacterium tuberculosis* (MTB) by culture requires up to 6 weeks, which has now resulted in PCR becoming established as a rapid method for early detection of MTB in developed and developing countries (Rodrigues and Vadwai, 2012; McNerney and Zumla, 2015). Rapid screening for sexually transmitted diseases by PCR for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* has been the front line testing strategy for over 10 years (Lee, Vigliotti and Pappu, 2009; Centers for Disease Control and Prevention., 2014). Recent developments in the diagnosis of sexually transmitted infections now include PCR for the detection of the flagellate parasite *Trichomonas vaginalis* (Šoba, Skvarč and Matičič, 2015) and difficult to culture bacterium *Mycoplasma genitalium* (Ross and Jensen, 2006). Probably the most recent transformation in the routine diagnostic laboratory is the detection of enteric pathogens from stool, with PCR replacing culture as the front line test in some laboratories (Koziel *et al*., 2013). It is fair to conclude that bacteriology is now undergoing a baseline shift in the methods used to routinely diagnose infection by incorporating PCR into both primary and secondary workflows.

So why are fungal diagnostics lagging behind in the introduction of molecular methods in support of the diagnosis of fungal infections?

What is known is that generally there is a very low burden of fungal DNA within clinical specimens; therefore PCR stands out as the most appropriate method to use when trying to detect fungi direct from these specimens (Loeffler *et al*., 2002). The main challenge faced by mycologists is the risk of environmental contamination, mainly with filamentous moulds (Alanio and Bretagne, 2014) but also *Candida*. False positives through the detection of colonising endogenous fungi are also of concern when using highly sensitive PCR assays. False positivity in *Aspergillus* PCR is a well-recognised complication when utilising PCR for the diagnosis of IFI (Alanio and Bretagne, 2014). Despite these complications PCR is being utilised in specialized mycology and reference laboratories for the diagnosis of fungal disease. In the Netherlands a survey

of 16 hospitals reported 12.5% (2/16) centers using PCR for fungal identification. In the UK a survey of 21 laboratories in 2004 reported that 28.6% (6/21) provided PCR as part of their diagnostic service (van der Linden *et al*., 2015). However, the respective survey was most likely subject to bias as the region surveyed has a concentration of specialist microbiology centers. A much larger survey of the UK published in 2008 revealed that the proportion of laboratories offering PCR as part of their routine diagnostic service was proportionally considerably lower, at 14% (9/64) reporting the use of PCR routinely (Barnes *et al*., 1996). An updated survey on the diagnostic landscape is now underway and it is anticipated publication will reveal an increase in the use of PCR for the diagnosis of IFI. The main utility across centers adopting PCR for diagnosis of IFI is for the detection of invasive aspergilllosis, invasive candidiasis and *Pneumocystis* pneumonia (PCP). PCR is also used as a pan-fungal approach for the identification of fungi from a variety of clinical specimens after fungal elements are detected following microscopic examination, most frequently from histopathological assessment of tissue sections. In a recent publication in Lancet Infectious Diseases the British Society for Medical Mycology (BSMM) published recommendations for the diagnosis of serious fungal infection and included PCR as diagnostic tests for patients at high risk of invasive fungal disease (Schelenz *et al*., 2015). It is clear that the challenge faced is taking PCR from being a specialist test offered from regional or national centres to one that can be used by most routine diagnostic laboratories as a standard of care. In the last decade the EAPCRI has worked to standardise PCR for the diagnosis of invasive aspergillosis from blood and BAL specimens. The same group is now applying this approach in an international effort called the Fungal PCR Initiative (FPCRI) for the standardization of PCR for the diagnosis of invasive candidiasis, *Pneumocystis* pneumonia, mucoraceous fungal infection and PCR for the identification of fungi from tissues.

### 1.12.3 Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

The term ‘matrix assisted’ laser desorption ionization mass spectrometry was first described by Hillenkamp *et al* in 1990 for the analysis of peptides and proteins (Hillenkamp and Karas, 1990). MALDI-TOF MS is capable of analysing a variety of cellular components including lipids, proteins and polysaccharides. The basic principles of MALDI-TOF MS are demonstrated in figure 1.22 (Vestal and Hayden, 2007; Carbonnelle *et al*., 2011; Croxatto, Prod’hom and Greub, 2012; Bourassa and Butler-

Wu, 2015);

1. Matrix: a chemical (suitable to the molecules being targeted) that is stable under normal atmospheric conditions that can be applied to an analyte and co-crystalizes, in the process charging the analyte by donating protons.
2. Laser desorption/ionisation; a laser is fired, inside a vacuum, at the matrix-analyte complex which liberates molecules into a vacuum chamber
3. Time of flight; based on the mass to charge ratio, molecules migrate through the vacuum chamber, directed by an electric field generator, towards the detector.
4. Mass spectrometry; as the analyte passes the detector a signal is created which is then interpreted by software to produce a mass spectrum that represents the analyte.

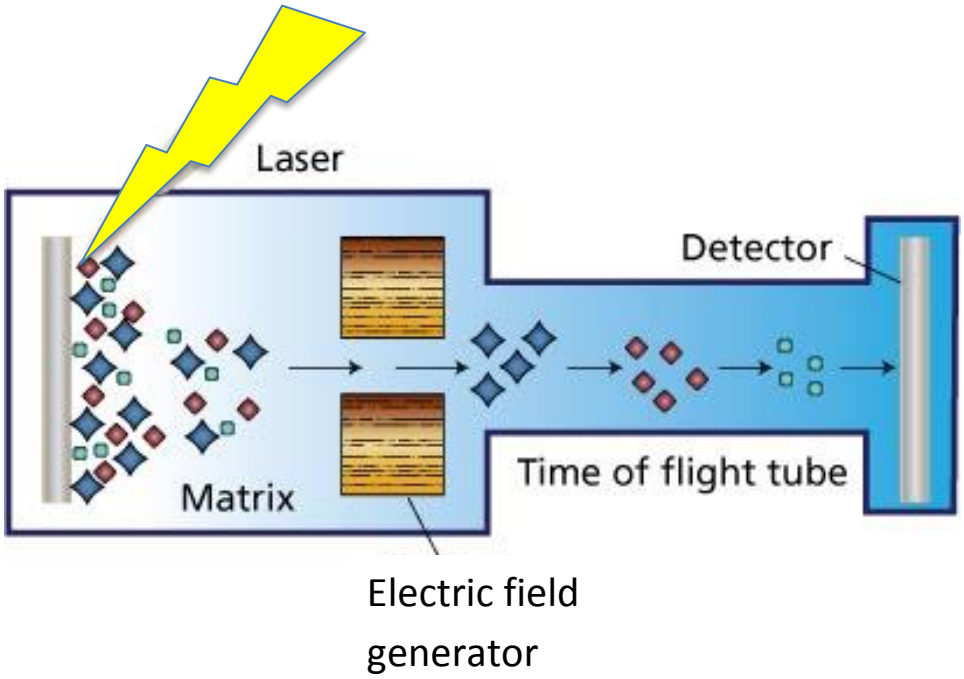



Figure 1.22 MALDI-TOF MS analysis demonstrating laser desorption of an analyte/matrix complex, with vaporisation into a vacuum chamber and time of flight of molecules based on their mass to charge (m/z) ratio through to a detector (slideshare.com).

In the early 1990's MALDI-TOF MS had evolved as a technology and its application in medical microbiology was underway. In 1996 Claydon and colleagues described the use of MALDI-TOF MS for the identification of bacteria from solid agar cultures (Claydon *et al*., 1996). The discriminatory power of MALDI-TOF MS was clearly demonstrated with the correct identification of several *Escherichia coli* and *Staphylococcus aureus* strains. Claydon described unique peaks for the respective

species, which allowed subsequent identification and established the basic principle of using mass spectra obtained by MALDI-TOF MS and peak selection for microbial identification (Claydon *et al*., 1996).

Up until this point the use of MALDI-TOF MS for microbial identification relied on the manual analysis of peak positions. The final leap in the development of this technology as a routine diagnostic tool came from the development of software capable of analysing and processing spectra and matching these unknown spectra to those contained within a reference database (Claydon *et al*., 1996). The proof of principle was established through initial studies that developed algorithmic approaches for matching MALDI-TOF MS spectral fingerprints to reference spectra in small databases initially containing five species represented by a single strain (Wunschel *et al*., 2005) or multiple strains of one species, *E. coli* (Arnold and Reilly, 1998). In both studies it was demonstrated that unknown isolates could identify against the reference database with high accuracy. In 2002 Bright and colleagues developed MUSE (Manchester Metropolitan University Search Engine), a reference database containing 35 strains, representing 20 species and 12 genera (Bright *et al*., 2002). This study was the first to demonstrate the use of software employing a spectral matching algorithm, enabling unknown spectra to be analyzed alongside a database containing several species and the correct identification be returned in the majority of analyses, in this case for 84% of the isolates. Other groups also demonstrated successful identification using in-house spectral databases (Bright *et al*., 2002).

In 2007, the first commercial bench top MALDI-TOF system, the Microflex alongside the first Biotyper database, was released by Bruker Daltonics. The Biotyper system was and still is centred on the analysis of whole cell and extorted proteins, mostly abundant ribosomal proteins, for the identification of microorganisms. The sentinel publication investigating the Biotyper system detailed the analysis of 1116 bacterial isolates, comparing identification with phenotypic bacterial identification systems and 16S rDNA sequencing to resolve discrepancies. 95.2% of the isolates were correctly identified with the MALDI-Biotyper. Differences in the identification rates for different groups of bacteria were noted with 95.5% success for enterobacteriaceae and 100% for staphylococci (Mellmann *et al*., 2008). The turnaround time to identification was reduced significantly from 18-24 hours to 12 minutes from culture. Over the last decade the application of MALDI-TOF MS for the identification of bacterial isolates has established itself as a conventional method used in many laboratories in developed countries with the financial resources to afford the technology. Over 200 studies are

now published (pubmed search parameters ((MALDI[TITLE]) OR MATRIX-ASSISTED[TITLE]) AND BACTERIA[TITLE]. Despite these advances, the use of MALDI-TOF MS for the identification of fungi is lagging behind with 34 studies listed for yeast identification (Pubmed search parameters 'MALDI[TITLE]) OR MATRIX-ASSISTED[TITLE]) AND YEAST[TITLE]') and 22 studies for the identification of filamentous fungi (Pubmed search parameters '(MALDI[TITLE]) OR MATRIX-ASSISTED[TITLE]) AND FUNGI[TITLE']. Hettick and colleagues published the first proof of principle study in 2008 created a reference database for 12 *Aspergillus* species (Hettick *et al*., 2008). Proteins were extracted using mechanical lysis in a trifluoracetic acid/acetonitrile suspension. Spectra obtained from the 12 species were cross analysed and no misidentifications occurred providing spectra contained a minimum of 28 significant peaks (Hettick *et al*., 2008). Some two years later an in-house reference database was created utilising a novel processing method to generate reference spectra known as the 'colony scrape' technique (Marinach *et al*., 2009). Retrospective analysis of 140 isolates matched against the database demonstrated a 98.6% identification success rate (Marinach *et al*., 2009). De Carolis and colleagues created an in-house database representing 55 fungal species of *Aspergillus*, *Fusarium* and *Mucorales*, using the same colony scrape method (De Carolis *et al*., 2012). The analysis of 103 blind-coded fungal isolates against this expanded database demonstrated 96.8% accuracy when identifying species contained within the reference database (De Carolis *et al*., 2012). All respective studies have demonstrated the strength of MALDI-TOF MS lies within its specificity when applied to fungal identification. This is in contrast to other molecular methods currently used for fungal identification, such as pan-fungal sequencing with the 18S rRNA gene, which lack in specificity (Balajee *et al*., 2009). The turnaround time to identification is an attractive feature as identifications can be achieved within 20 minutes from a colony compared with up to 2 days for pan-fungal sequencing methods. MALDI-TOF MS methodology is also more cost effective when amortised over a prolonged period, with an average MALDI-TOF MS analysis costing less than £1.00 per analysis (Ge *et al*., 2017). The development of MALDI-TOF MS methods is required as many studies detail the use of in-house databases, which limits the routine use of MALDI-TOF MS, as the databases are not made freely available. Processing methods are not standardised with several different approaches to protein extraction published.

### 1.12.4 Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH)

In 1977 Cheung and colleagues described a novel method for the mapping of mammalian genes, utilising *In situ* hybridization of mRNA to chromosomal DNA

(Cheung *et al*., 1977). By applying DNA probes to immobilized mammalian cell preparations on a slide the resultant mRNA/DNA hybridization product could be visualized by fluorescence microscopy *in situ*. This was the first description of FISH for visualization of nucleic acid. Over 40,000 publications have now detailed the use of this technique for a variety of applications, most commonly in the study of chromosomes in cytogenetic applications.

Fluorescence *in situ* hybridisation (FISH) for the identification of yeasts was first described by Lishchewski and colleagues who used a 20-nucleotide DNA probe complementary to a region of the 18S ribosomal gene labeled with a 5(6)-carboxy-fluorescein-A-hydroxysuccinimide ester The probe targeted *C. albicans* and *C. tropicalis.* Both species could be differentiated from other commonly encountered *Candida* species using the probe and in the presence of human epithelial cells cross-reaction did not occur with human DNA (figure 1.23). The turnaround time to results was less than one day, far quicker than other molecular methods available at this time (Lischewski *et al*., 1996). This preliminary work led to the successful application of FISH for the identification of yeasts within paraffin embedded sections of tissue from murine kidney and brain infected with *C. albicans* (Lischewski *et al*., 1996)

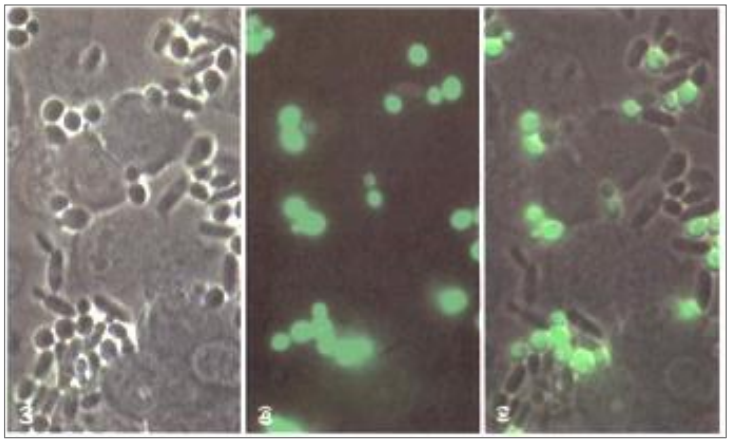


Figure 1.23 (a) Phase contrast micrograph showing the round C. *albicans* and the oval-shaped C. *krusei* adhering to the endothelial cells. (b) The same section viewed by fluorescence microscopy. (c) Double exposure of the phase contrast and fluorescence micrographs. Note that only the round C. *albicans* cells, but not the longer C. *krusei* cells are stained by the fluorescently labelled probe 020 (Lischewski *et al*., 1996).

Some two years later Kempf and colleagues demonstrated the first application of FISH for the identification of microorganisms direct from positive blood cultures (Kempf *et al*., 2000). Using a panel of DNA probes targeting bacterial and fungal pathogens, known

to cause 95% of blood stream infections in their setting, they demonstrated that 96.5% of microorganisms within blood cultures could be identified in less than 2.5 hours of blood cultures becoming positive (Kempf *et al*., 2000). Probes for *C. albicans*, *C. glabrata, C. krusei* and *C. parapsilosis* were included with no reported cross-reaction of probes and the successful identification of two *C. albicans* fungaemia episodes in real time (Kempf *et al*., 2000). DNA probes had been shown to be successful yet efficiency of probe hybridization is reliant on optimal sample preparation. DNA probes have a negative charge and are therefore subject to repulsion from negatively charged, or adherence to positively charged molecules, that may be present within the cell wall of microorganisms. It is necessary to use ethanol fixation, plus enzymes such as lysozyme for *S. aureus*, to make the cell membrane permeable whilst retaining morphological features, to allow successful hybridization of the probe (Lischewski *et al*., 1996; Kempf *et al*., 2000). Researchers working in this field realized that neutralization of the negative charge associated with probes would enable more efficient probe hybridisation.

In 1993 Egholm and colleagues described the design of a novel DNA analogue, peptide nucleic acid (PNA), in which the phosphate backbone of DNA is replaced with a structurally homomorphous backbone consisting of N-(2-aminoethyl) glycine units to which nucleotides are attached, figure 1.24. The resulting PNA molecules carrying a neutral charge, bound to complementary nucleotides with greater stability compared to the DNA probes (Nielsen, Egholm and Buchardt, 1994).

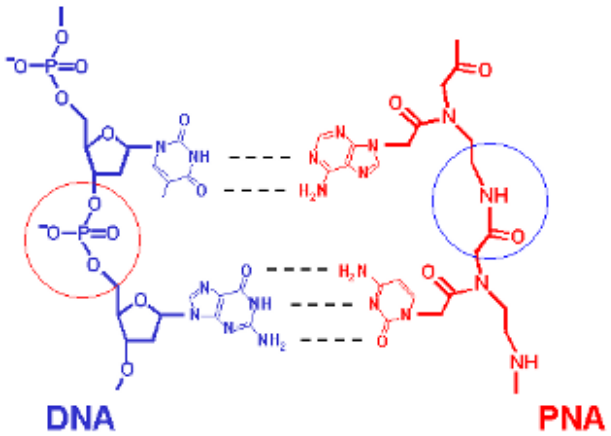



Figure 1.24 Peptide nucleic acid (PNA) molecule bound to DNA demonstrating the neutrally charged peptide substitution in the backbone replacing the negatively charged phosphate molecule (Mateo-Mart and Pradier, 2010).

The neutrally charged backbone of PNA molecules no longer affected by repulsion between two negatively charged DNA molecules facilitated stronger bonding of the probe to its target inside the cell (Nielsen, Egholm and Buchardt, 1994; Mateo-Mart and Pradier, 2010). Figure 1.25 demonstrates the PNA binding process to RNA molecules within whole cells, in this example staphylococcus cells, and the overall fluorescence omitted from cells where a red fluorescing probe has bound to the target RNA molecule (Jensen *et al*., 2014). Oliveria *et al* designed the first PNA probes for the identification of yeasts from liquid culture. In this study two probes targeting the 26S rRNA were designed for the closely related species *C. albicans* and *C. dubliniensis*. Processing time was 1-3 hours dependent on the fixing method utilised and 100% sensitivity and specificity of the two PNA probes was observed when tested against multiple isolates of the two respective species (Oliveira *et al*., 2001). In a further study the same group then applied the *C. albicans* probe to the prospective analysis of positive blood cultures and further tested the probe for cross reaction with other *Candida* species. Again 100% sensitivity and 100% specificity was observed when testing the probe against 18 different species across 229 analyses in the presence of human blood (Rigby *et al*., 2002).

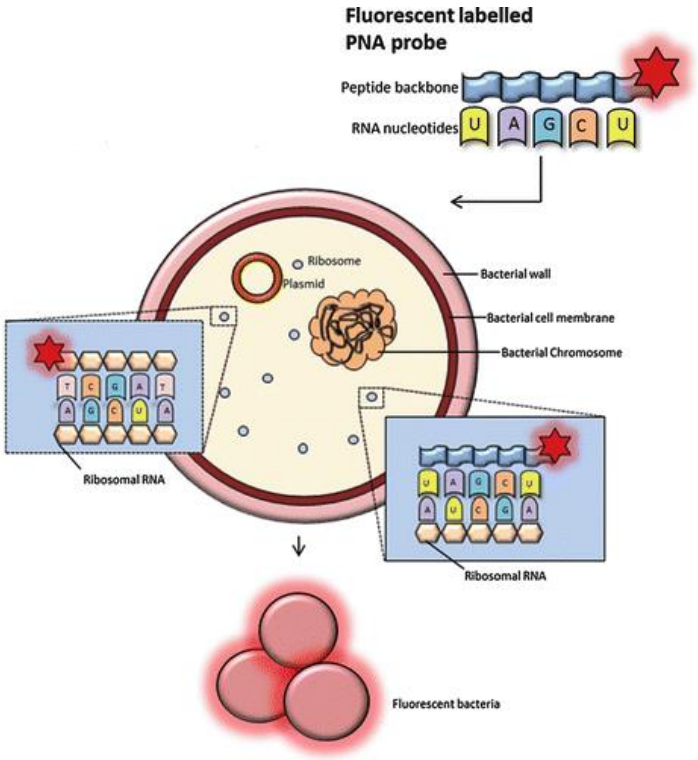



Figure 1.25 Intracellular fluorescent *In situ* hybridisation using PNA probes complementary to ribosomal RNA molecules (Jensen *et al*., 2014).

### 1.12.5 Clinical laboratory mycology diagnostic service

At the time of study the diagnostic service at the Royal Free Hospital microbiology department included tests outlined in figure 1.26. The workflows are a fair representation of many clinical diagnostic laboratories to date (Barnes *et al*., 1996, Schelenz *et al*., 2015). Blood cultures, if positive, were processed with Gram's stain, culture and biochemical identification using the AuxaColor 2 assay. Galactomannan was performed twice weekly for patients at high risk of IA and also as a diagnostic test for patients with a high suspicion of IA. BAL for *Pneumocystis* pneumonia was processed by cytology using GMS staining. For the investigation of Invasive mould infection Calcofluor was performed on BAL or tissue followed by extended culture (3 weeks) for filamentous moulds. If positive, conventional identification of moulds was performed using microscopy. If identification was unsuccessful isolates were referred to the reference laboratory.

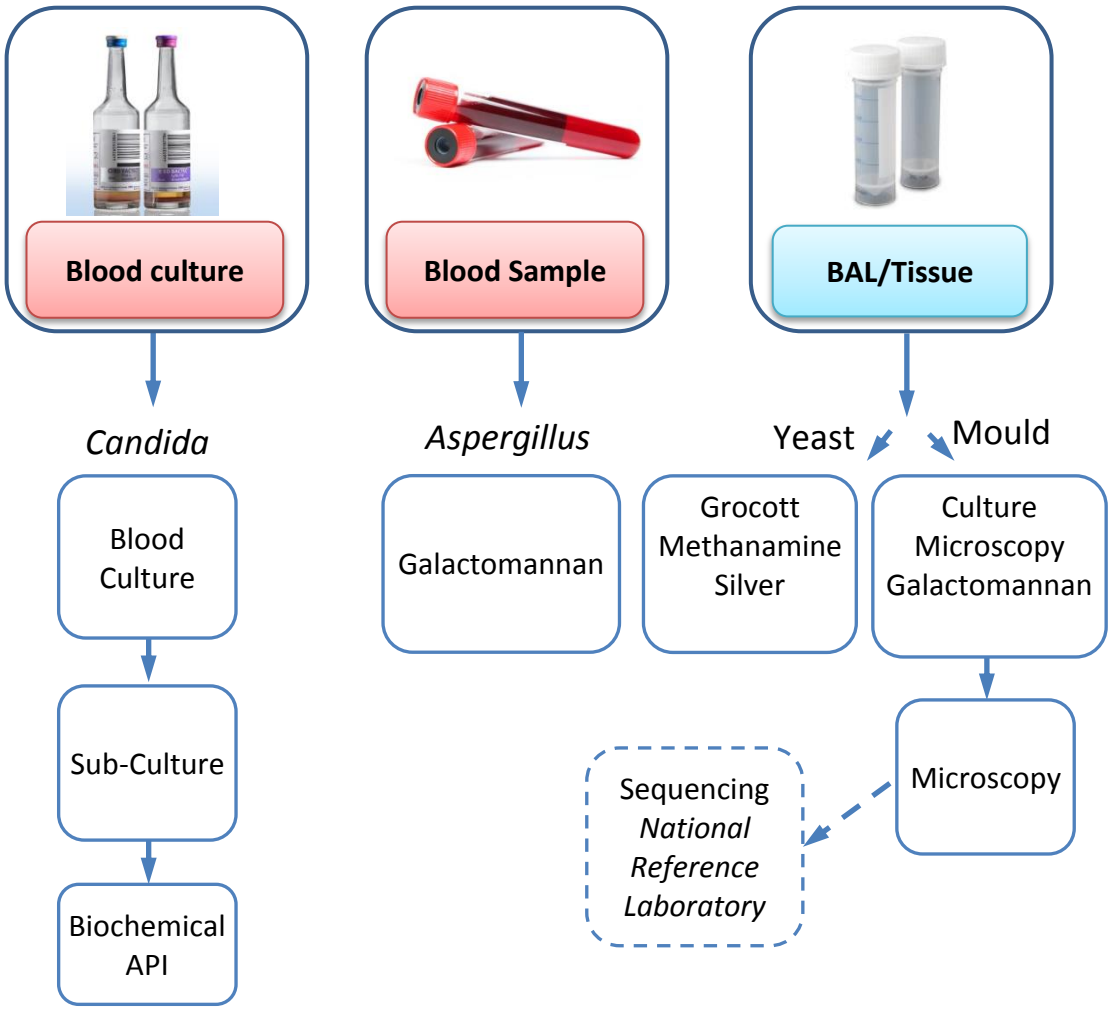



Figure 1.26 Routine Diagnostic Service at the Royal Free Hospital diagnostic microbiology laboratory – 2009.

### 1.13 Research Aims

The aims of this research are to comprehensively evaluate the use of molecular diagnostics to address the current shortfall in the diagnosis of IFI and where appropriate embed new methods and technology into the clinical service.

IFI targeted in the research are invasive candidiasis, invasive aspergillosis, *Pneumocystis* pneumonia and other non-*Aspergillus* invasive mould infections. The respective invasive fungal infections have diagnostic challenges unique to the pathology of each disease and the patient cohorts who are susceptible to each IFI. The limitations of current diagnostic techniques were assessed so that the most appropriate molecular techniques could be applied to the diagnostic scenario for each IFI to investigate if diagnosis could be improved.

A range of molecular methodologies, including polymerase chain reaction (PCR) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) and peptide nucleic acid fluorescence *In situ* hybridisation (FISH), were evaluated on clinical sample cohorts established from pathology samples surplus to diagnostic requirements from the Royal Free Hospital clinical pathology archive. Each method evaluated was critically appraised for; statistical performance, clinical utility and suitability for service adoption.

This research was undertaken as part of the National Institute for Health Research NIHR/Chief Scientific Officer (CSO) Healthcare Science Research Fellowship. The aim of the program was to support the development of research infrastructure, capacity and capability in healthcare science. The intention of research undertaken throughout the time of the award was to support this research in the NHS setting bridging the gap between clinical/service careers and research at doctoral level through translational research.

# Chapter 2 General methods

## 2.1 Culture based methods

### 2.1.1 Subculture of yeast isolates

Yeasts stored in water were vortexed for 10 seconds prior to subculture to re-suspend the isolate. A 10µL loop of the *Candida* suspension was streaked out onto CHROMagar and incubated at 36°C for 24 h. Subcultures were passaged twice to ensure purity. (Becton Dickinson, Franklin Lakes, NJ, and USA).

### 2.1.2 Biochemical identification of yeasts

Apple green colonies on CHROMagar *Candida* (Becton Dickinson, New Jersey) were identified as *C. albicans.* All non-albicans *Candida* species were subject to testing with the Auxa*Color* 2 assay (Bio-Rad, California) as per manufacturer's instructions. Cornmeal agar slide preparations (supplemented with 1% Tween 80 and a sterile cover slip placed on the agar square) were used to establish morphological characteristics for the complete species profile.

### 2.1.3 Simulated blood cultures

The spiking inoculum was prepared by making a 0.5 McFarland suspension of the yeast isolates in saline, representing approximately $10^8$ cfu/mL. 10µL of the suspension was added to 10mL of saline and vortexed to make suspension 2. 100µL of suspension 2 was transferred to 900µL saline to make suspension 3.

Anonymised blood culture bottles, determined to be negative after 5 days incubation on the BacTec, were spiked with 10µL of suspension 3 of each yeast isolate containing approximately 100–200 cfu. Spiked blood cultures were re-incubated on the BacTec system (Becton Dickenson, New Jersey) until positivity was detected. An inoculum of 10µL was plated onto CHROMagar *Candida* medium to obtain an approximate colony count. Bottles were spiked in batches of 10. At the point of positivity blood cultures were subjected to Gram's stain and CHROMagar *Candida* subculture to confirm purity.

### 2.1.4 Mycelial mat culture for filamentous moulds

To create mycelial mats for DNA extraction 3cm microtiter plates were used. One plate per isolate was prepared. 5mL of Sabouraud's broth was inoculated into the wells of the microtiter plate. Using a wet cotton swab the fungal colony was gently brushed then

the harvested spores were transferred into a microtiter well containing Sabouraud's broth. The lid was fixed into position and plates were incubated in a shaking incubator at 30°C for 24 hours, or until a flat mycelium mat had formed. The mycelial mat was transferred into a 5mL bijou tube and frozen at -20°C until required for DNA extraction.

### 2.1.5 Miles and Misra

Miles and Misra counts on positive blood cultures were prepared from serial dilutions of the blood culture. 100µL of positive blood culture was aspirated and added to 900µL of saline. The tube was vortexed for 10 seconds. Using a fresh pipette tip 100µL of the $10^{-1}$ dilution was transferred to another 900µL of saline and vortexed to make the $10^{-2}$ dilution. The dilution process was repeated to obtain serial dilutions up to a $10^{-7}$ dilution. 100µL of each dilution was plated out onto a CHROMagar *Candida* plate and incubated at 35°C for 24 hours. Colony counts were performed from the two dilutions that had distinct colonies. An average cfu/mL was calculated from a mean of the two counts, i.e. if the $10^{-4}$ count was 180 and the $10^{-5}$ count was 16 the mean = 1.7 x$10^{6}$

## 2.2 Molecular based methods

### 2.2.1 DNA extraction from yeasts and mycelial mats

For yeast isolates a 4.0 McFarland suspension of each isolate was prepared in molecular grade water. For the mycelial mats a small pea sized piece of mycelium was added to 300ul of sterile distilled water. Acid washed glass beads (~50µL equivalent) were added to the fungal suspensions. Ribolysis was performed for 3 minutes at 6.5m/s speed using a MP Fast-prep 24 platform. Lysates were processed using the Wizard genomic DNA kit (Promega, Wisconsin, USA) as per manufacturer's instructions for yeast cells (omitting the lyticase treatment step as mechanical lysis was adequate for fungal cell wall lysis).

### 2.2.2 Gel electrophoresis for detection of PCR amplification

PCR products were separated on a 1.5% agarose gel and DNA bands were visualized with ethidium bromide. A 100bp ladder was used to size products.

### 2.2.3 Big dye terminator sequencing

PCR amplicon clean up was performed using a kit as per manufacturers instructions (Qiagen) designed to remove excess nucleotides and primers from the amplified DNA. Sequencing was performed in duplicate in the forward and reverse direction. The reaction mix consisted of 5µL template DNA, 2µL of PCR primer, 8µL water, 2µL BigDye buffer and 2µL BigDye Terminator v3.1 Ready Reaction Mix (Applied

Biosystems). Reaction cycling was as follows: 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 seconds. Ethanol precipitation clean up was used to purify sequencing products. The pellet of DNA was air-dried and rehydrated in 15µL formamide. Analysis was performed on a 3130 Genetic Analyser Capillary Array for detection (Applied Biosystems). Bionumerics v5.1 (Applied Maths) was used to obtain a composite sequence. Fungi were identified for each assay by comparing consensus sequences to a database library of known ITS rRNA gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) by multiple sequence alignment (Jenkins *et al.*, 2012).

### 2.2.4 Ribosomal rRNA ITS amplification and sequencing

Amplification of the ITS1-5.8S-ITS2 region was performed using previously described ITS primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3 (Innis MA, Gelfand DH, 1990). PCR was performed in 50µl reactions in the presence of 2U Hotstar Taq polymerase, 1.5mM MgCl2, 0.3mM of each deoxynucleoside triphosphate, 0.5mM of each primer and 5ul of template DNA. Following enzyme activation at 94ºC for 15 minutes reactions were subject to 40 thermal cycles of 94ºC (60s), 50ºC (60s) and 72ºC (180s) on a GeneAmp® PCR system 9700 thermocycler. DNA amplification was detected by electrophoresis of a fraction of the amplification product on a 0.5% (wt/vol) agarose gel run for 30 minutes at 170V in 0.5 x Tris-borate buffer. Sequencing was performed as detailed by Jenkins *et al* (2012).

## 2.3 MALDI-TOF MS methods

### 2.3.1 Conventional formic acid extraction

A 1 µL loop full of yeast colony was suspended in 300µL of HPLC grade water then 900µL of absolute ethanol was added. After centrifugation the supernatant was removed and the pellet air-dried for 10 minutes. Pellets were re-suspended in 40µL of 70% formic acid and incubated for 2 minutes. 40µL of acetonitrile was added and the solution was thoroughly vortexed before a final centrifugation at 8000g for two minutes. 1µL of supernatant was used for analysis and spotted in duplicate. 1µL α-Cyano-4-hydroxy-cinnamic-acid matrix was immediately added to each spot once dry. Time to result was measured for a single isolate for the whole identification process including software analysis.

### 2.3.2 MALDI-TOF analysis

Spectra acquisition was performed on a Microflex™ LT platform (Bruker Daltonics) with Flex Control software (version 3.0) using default settings. Spectra analysis was performed using MALDI Flex analysis™ software analysing spectra in the 2000-20,000 m/z range. Log similarity score thresholds for the MALDI Biotyper identifications were defined as; no reliable ID 0-1.699, probable genus 1.7-1.999, secure genus, secure species >2.0. Re-analysis of spectra was also performed lowering the species acceptable log similarity score threshold to >1.9.

### 2.3.3 Bruker log similarity score calculation

The number of peaks matched between the sample and the reference spectra divided by the overall number of peaks in the samples to gives a score out of 10. Followed by the number of peaks matched in the unknown sample is divided by the number of peaks in the reference sample to give a score out of 10. Finally the relative intensities of the matching peaks are calculated to give a score out of ten. These three values are multiplied in sequence to give a score out of 1000 and the log of that score if then calculated to give a score out of 3. In the example below the calculation is as follows:

1. There are six reference peaks in the unknown spectra containing 20 peaks = 6/20
2. There are six peaks matched in the unknown spectra divided by the 10 peaks in the reference spectra = 6/10
3. Then the score of the relative intensities of the matched peaks is calculated

   The final score = 6 x 3 x 7 = 126 = log similarity score 2.1

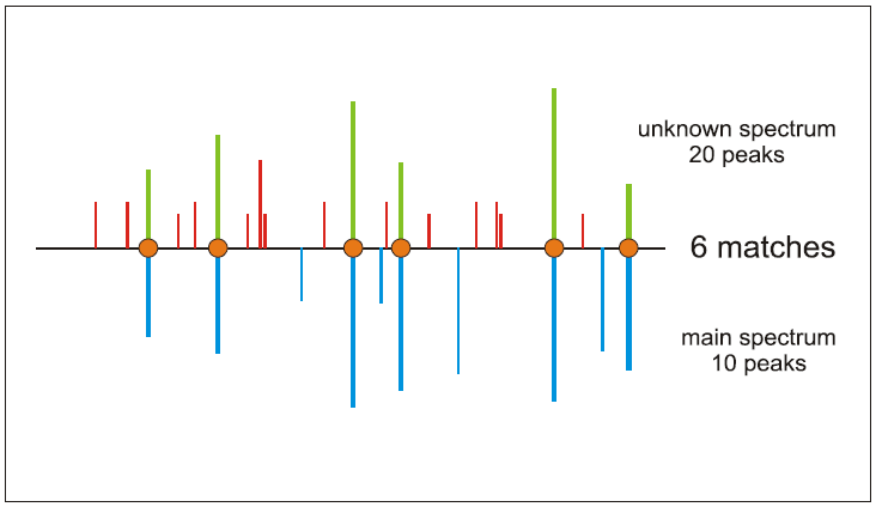



Figure 2.1 example of spectral matching with unknown spectra (top) matched against reference spectra (bottom). Peak matching and unmatched peaks are used to calculate the log similarity score.

### 2.3.4 Reference laboratory identification of fungi

Isolates requiring further identification were referred to Bristol PHE MRL for processing on SAB slopes. ITS pyrosequenced based identification is utilised to identify fungi (Borman *et al.*, 2010).

# Chapter 3 Evaluation of solid agar culture processing methods and MALDI-TOF MS for the identification of clinically relevant yeast isolates

## 3.1 Introduction

Phenotypic and biochemical identification methods for yeast isolates, including sub-culture onto chromogenic media and biochemical profiling, are the conventional methods of choice in many diagnostic laboratories in the UK and globally (Davey *et al.*, 1995; Romney *et al.*, 2000; Meletiadis *et al.*, 2011). Using chromogenic agar the turnaround time (TAT) for identification is 24 hours for *C. albicans*. An additional 48-72 hours is required to achieve identification for all other yeast species using biochemical assays, such as the Auxa*Color* 2 (Bio-Rad, California) (Meletiadis *et al.*, 2011). One fundamental drawback is that commercially available biochemical assays are supported by databases that are limited in their species coverage leading to misidentification of rare yeasts such as *C. famata*, *C. guilliermondii*, *C. lusitaniae* (Posteraro *et al.*, 2013), as discussed in section 1.5.5 of the introduction (Massonet *et al.*, 2004; Kullberg and Arendrup, 2015). Some yeasts, such as *Candida famata*, may also remain unidentified, as their standard biochemical profiles obtained using commercially available assays cannot be interpreted. This contributes to a delay in achieving a full diagnosis which may have a subsequent impact on the clinical management of the patient (Sheppard *et al.*, 1998;; Ullmann, *et al.*, 2011). A meta-analysis evaluating the accuracy of biochemical tests for yeast identification concluded the pooled identification ratio at the species level to be 0.89 (95% CI: 0.8-0.98) (Posteraro *et al.*, 2013). Commercially available identification systems for bacterial identification have demonstrated similar rates of identification, 92.5% using the Phoenix and 88.8% using the Vitek system (Chatzigeorgiou et al., 2010). Therefore, there is a requirement across microbiology to improve the accuracy of identifying microorganisms. As more than 100 yeast and yeast-like species are known to be human pathogens (Ashbee *et al*., 2010) the identification of rarer yeast species presents a challenge for non-specialist diagnostic laboratories. The incidence of rare yeast species causing fungaemia is estimated at 1.8-3% (Arendrup *et al.*, 2008; Chitasombat *et al.*, 2012)

The Biotyper version 3.1 database (Bruker Daltonics, Germany) is a comprehensive data repository, containing reference spectra for >100 yeast species used in conjunction with MALDI-TOF spectroscopy. Prior to MALDI-TOF MS analysis it is recommended that yeasts undergo formic acid protein extraction (Bruker protocols). This is necessary as fungi have a robust fungal cell wall, comprised of glucan, mannan and chitins (Bowman and Free, 2006). The extraction process extends the turnaround time to results by 10-15 minutes (Bruker protocols) On an individual sample basis the increase in TAT is not significant but in a high throughput diagnostic setting it could contribute to a significant increase in the turn around time of results. A reduction in the time for this step may be achieved by application of formic acid directly onto yeast analytes applied onto the MALDI-TOF MS target plate, termed 'on-plate extraction', prior to the addition of matrix. At the time of study this was suggested by Bruker as an alternative method for yeast identification but was unpublished. The aim of this study was to evaluate the Microflex (Bruker Daltonics, Germany) and Biotyper V3.1 software (Bruker Daltonics, Germany) for the identification of clinical yeast isolates from solid agar cultures. Both conventional and on-plate formic acid extraction methods were investigated and compared with a gold standard ITS rRNA sequence analysis (Innis MA, Gelfand DH, 1990) and previously obtained biochemical identifications using the Auxa*Color* 2 (Bio-Rad, California) assay.

## 3.2 Methods

### 3.2.1 Yeast isolates

Clinical isolates classified as surplus to diagnostic requirements were selected from the clinical archive of isolates stored by the UK CMN regional laboratory at the Royal Free Hospital NHS microbiology department (Pond Street, Hampstead, London) and were accessed under local clinical service improvement guidelines; isolates were stored in water away from light (Borman *et al.*, 2006).

### 3.2.2 Subculture of isolates

See general methods section 2.1.1

### 3.2.3 Biochemical identification

See general methods section 2.1.2

### 3.2.4 DNA extraction

See general methods section 2.2.1

### 3.2.5 Ribosomal rRNA ITS amplification and sequencing

See general methods section 2.2.2 - 2.2.4

### 3.2.6 Conventional direct analysis

For direct analysis a small amount of a single yeast colony was inoculated in duplicate spots onto the target plate in thin smears using a wooden mixing stick (without returning to the culture for the second spot to enable a lighter smear). 1 µL of α-cyano-4-hydroxy-cinnamic-acid matrix was pipetted onto each spot. For yeasts failing to identify on first attempt a repeat analysis was performed.

### 3.2.7 On-plate FA extraction

For on-plate FA extraction a small amount of a single yeast colony was inoculated in duplicate spots onto the target plate in thin smears using a wooden applicator (without returning to the culture for the second spot so enable a lighter smear). 1µL of 100% FA was pipetted onto each spot, allowed to dry then 1µL of α-cyano-4-hydroxy-cinnamic-acid matrix was immediately added to each spot.

### 3.2.8 Conventional Formic Acid extraction

See general methods 2.3.1

### 3.2.9 MALDI-TOF MS analysis

See general methods 2.3.2

### 3.2.10 Log similarity score manipulation

Stepwise reduction in the log similarity score was investigated to assess the impact on the rate of identification using the different identification methods, measures of >2.0, >1.9, >1.8 and >1.7 were evaluated as acceptable thresholds for species identification. Lowering the log similarity score was previously described by Van Herendaelan and colleagues (2012) as an effective method for increasing the success of MALDI-TOF for yeast identification (Van Herendael *et al.*, 2012). Identifications were only considered accurate if complete agreement was obtained with ITS rRNA sequenced based identification for the top three identifications returned by the biotyper software.

### 3.2.11 Statistical analysis

Calculation of the log similarity score by the Biotyper 3.1 software (Bruker Daltonics, Germany) can be found in section 2.5 of the general methods section. To calculate if the difference between the rates of identification was significant or not across the different processing methods an "N-1" Chi-squared test was applied as recommended by Campbell (2007) and Richardson (2011). MedCalc (medcalc.org, Belgium) software was used to perform this test.

### 3.2.12 Time to identification

The time to identification of a single yeast isolate was measured by timing the same operator performing on plate formic acid extraction and conventional formic acid extraction, from the start of the process to the final identification.

### 3.2.13 Prospective yeast identification performance

Yeast identification by MALDI-TOF MS in the clinical diagnostic service was implemented in January 2012. Figure 3.3 demonstrates the workflow for yeast MALDI-TOF MS analysis. On-plate formic acid extraction was used as first attempt processing for yeast isolates, with conventional formic acid extraction being performed on isolates failing to identify. A species log similarity score threshold of >1.9 was used for identification.

## 3.3 Results

### 3.3.1 Yeast isolates

Clinical yeast isolates (n=190) were collected over a 12-month period from 1$^{st}$ January 2012 to 31$^{st}$ December 2012. Table 3.1 details the ITS rRNA sequence based identifications from the cohort. In total, 19 yeast species were represented. Commonly encountered species *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. guilliermondii* accounted for 87.9% (167/190) whilst 12.1% (23/190) of the yeast isolates were rarely encountered yeast species.

### 3.3.2 Performance of MALDI-TOF MS processing methods

The performance of each processing method for the identification of yeasts was calculated from the first attempt at identification using each method.

Repeat analysis was performed for yeast isolates that hadn't identified on first attempt using conventional formic acid extraction. The overall identification rate, after repeat analysis, is presented in table 3.2.

**Table 3.1. ITS rRNA sequenced based identifications for 190 yeast isolates included within the study**

| ***Species*** | **%** | ***n*** | ***Species*** | **%** | ***n*** |
|---|---|---|---|---|---|
| *Candida parapsilosis* | 29.5 | 56 | *Saccharomyces cerevisiae* | 1.1 | 2 |
| *Candida albicans* | 26.3 | 50 | *Pichia fermentans* | 0.5 | 2 |
| *Candida glabrata* | 14.7 | 28 | *Candida lipolytica* | 0.5 | 1 |
| *Candida tropicalis* | 6.3 | 12 | *Cryptococcus neoformans* | 0.5 | 1 |
| *Candida guilliermondii* | 6.3 | 12 | *Candida nivariensis* | 0.5 | 1 |
| *Candida krusei* | 4.7 | 9 | *Pichia cactophila* | 0.5 | 1 |
| *Candida lusitaniae* | 1.6 | 3 | *Candida dubliniensis* | 0.5 | 1 |
| *Candida kefyr* | 1.6 | 3 | *Magnusiomyces capitatus* | 0.5 | 1 |
| *Pichia caribica* | 1.6 | 3 | *Cyberlindnera fabianii* | 0.5 | 1 |
| *Candida orthopsilosis* | 1.1 | 2 | *Debaryomyces nepalensis* | 1.1 | 1 |

### 3.3.3 Direct on-plate analysis from culture

One isolate was identified by direct preparation, as *C. guilliermondii* (log similarity score 2.04). For 21.6% (41/190) of the isolates a result of 'no reliable identification' was retuned. The median log similarity score for isolates returning either identification or a result of 'no reliable identification' was 1.332 (IQR: 1.281, 1.421), figure 3.1. For 77.9% (148/190) of the isolates no peaks were detected by MALDI-TOF therefore no log similarity score was generated.

### 3.3.4 Direct on-plate formic acid extraction

Using direct on plate formic acid extraction 41% (78/190) of yeast isolates were identified to species level with a log similarity score >2.0. A genus level identification was obtained for a further 33.7% (64/190) of the isolates with a log similarity score 1.7-1.999. A result of 'no reliable identification' was obtained for 25.3% (48/190) of the

isolates with a log similarity score <1.7. The overall median log similarity score obtained using direct on plate formic acid extraction was 1.97 (IQR: 1.84, 2.1), figure 3.1.

### 3.3.5 Conventional formic acid extraction

Using conventional formic acid extraction 83.7% (159/190) of yeasts were identified at species level with a log similarity score >2.0 and 13.7% (26/190) at the genus level with a log similarity score between 1.7-1.99 on first attempt. A result of 'no reliable identification' was returned for 2.6% (5/190) of analytes with a log similarity score <1.7. The overall median log similarity score obtained using conventional formic acid extraction was 2.19 (IQR: 2.07. 2.33), figure 3.1.

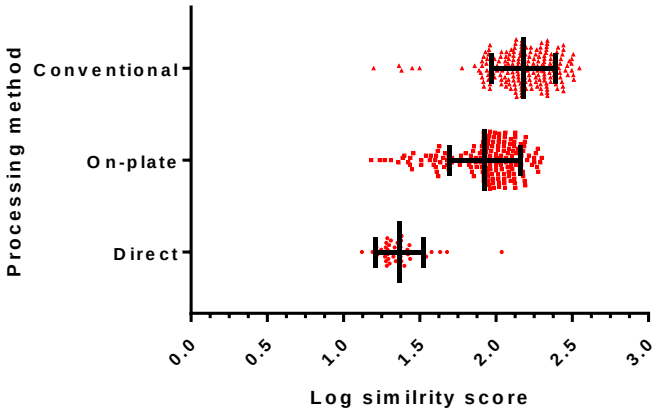



Figure 3.1 Log similarity scores obtained for 190 yeast isolates processed by direct smear, on plate and conventional formic acid extraction.

### 3.3.6 Log similarity score reduction for on plate formic acid extraction

By reducing the log similarity score species threshold to >1.9 a significant increase in the rate of identification ($p<0.001$) was observed for yeasts at the species level 63.7% (121/190). Further reducing the log similarity score to >1.8 increased the identification rate to 71% (135/190) but this was not a significant increase ($p=0.13$) compared to accepting a log similarity score >1.9. By further reducing the log similarity score threshold for identification to >1.7 the rate of identification increased further to 74.7% (142/190) but was not significantly different from the rate of identification with a log similarity score threshold of >1.9 ($p=0.02$) (table 3.2).

### 3.3.7 Log similarity score reduction for conventional formic acid extraction

By lowering the species log similarity score threshold to >1.9 the success rate for conventional FA extraction the identification rate increased to 90% (171/190) but was not significantly different (p=0.07) to the identification rate with a log similarity score >2.0. By lowering the species threshold to >1.8 the identification rate increased significantly (p<0.01) to 96.3% (183/190) compared with a log similarity score of >2.0 with no misidentifications compared to ITS rRNA sequencing. By reducing the log similarity score to >1.7 the identification rate increased to 97.4% (185/190) but was not statistically significant (p=0.54) compared with a log similarity score of >1.8.

**Table 3.2. MALDI-TOF MS and biochemical yeast identification success rates compared with ITS rRNA sequence analysis**

| **Processing method** | **Species** | | **Genus** | | **No ID** | | **Incorrect** | |
|---|---|---|---|---|---|---|---|---|
| **Conventional formic acid** | **%** | ***n*** | **%** | ***n*** | **%** | ***n*** | **%** | ***n*** |
| Log similarity score >2.0 | 83.7% | 159 | 13.7% | 26 | 2.6% | 5 | 0 | |
| Log similarity score >1.9 | 90% | 171 | 7.4% | 14 | 2.6% | 5 | 0 | |
| Log similarity score >1.8 | 96.3% | 183 | 1.1% | 2 | 2.6% | 5 | 0 | |
| Log similarity score >1.7 | 97.4% | 185 | 0% | 0 | 2.6% | 5 | 0 | |
| On-plate formic acid | | | | | | | | |
| Log similarity score >2.0 | 41.1% | 78 | 33.7% | 64 | 25.3% | 48 | 0 | |
| Log similarity score >1.9 | 63.7% | 121 | 11% | 21 | 25.3% | 48 | 0 | |
| Log similarity score >1.8 | 71% | 135 | 3.7% | 7 | 25.3% | 48 | 0 | |
| Log similarity score >1.7 | 74.7% | 142 | 0% | 0 | 25.3% | 48 | 0 | |
| Overall success | | | | | | | | |
| Conventional formic acid | 97.4% | 185 | 0 | | 2.6% | 5 | 0 | |
| Biochemical identification* | 94.2% | 179 | 0 | | 0 | | 5.8% | 11 |

**Auxa*color 2 identification (Biorad, California)

### 3.3.8 Unidentified yeasts by MALDI-TOF MS

After repeat analysis 2.6% (5/190) of the yeast isolates returned a result of 'no identification' with a log similarity score <1.7. Analysis of the MALDI-TOF MS spectra for these isolates demonstrated high quality spectra with a significant number of peaks at the peak intensity expected of an analysis that would have provided isolate identification. This is exemplified in figure 3.2 where 3.2a shows the spectra for a yeast that failed to identify compared with the spectra of a yeast in figure 3.2b, which

achieved a successful identification.

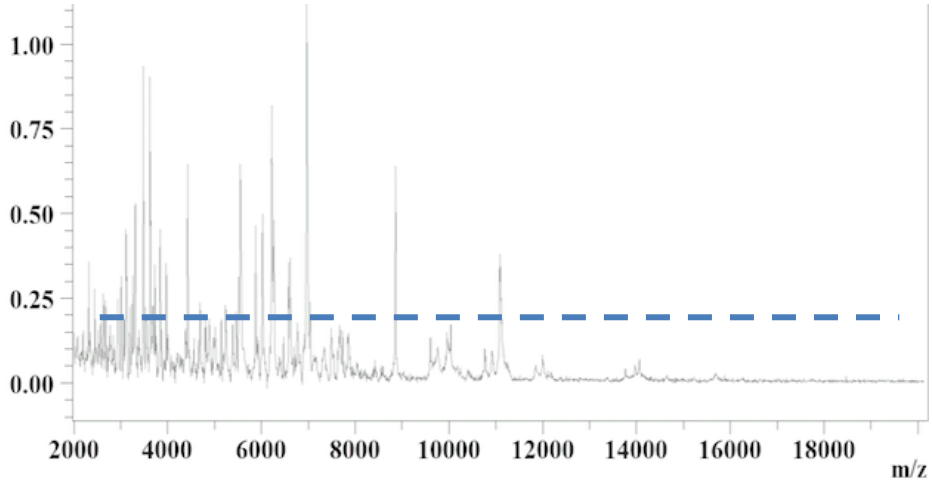



Figure 3.2 MALDI-TOF MS spectra obtained for a yeast isolate with a result of 'no-identification'. The threshold for peak acceptance is indicated by the dotted line. Peak intensity and abundance indicates this MS spectra should have resulted in a successful identification if a matching spectra was present in the reference spectral database.

### 3.3.9 Time to results

On-plate FA extraction had a turnaround time to result of 6 minutes for a single isolate. Conventional FA extraction had a turnaround time of 13 minutes for a single isolate.

### 3.3.10 Agreement between methods

Agreement between the three methods for the 190 isolates is detailed in table 3.3. The agreement between MALDI-TOF MS and ITS rRNA sequencing was 97.4% (185/190). Agreement between Biochemical identification and ITS rRNA sequencing was 94.2% (179/190). Agreement between MALDI-TOF and biochemical identification was 94.2% (179/190) agreement was achieved when compared with conventional identification methods. The difference in the rate of agreement was not statistically significant (p=0.12) across the three analyses.

**Table 3.3 Agreement between MALDI-TOF, ITS rRNA and Biochemical methods for the identification of 190 yeast isolates**

| **Methods** | **%** | ***n*** |
|---|---|---|
| MALDI-TOF & ITS rRNA | 97.4% | 185/190 |
| Biochemical & ITS rRNA | 94.2% | 179/190 |
| MALDI-TOF & Biochemical | 94.2% | 179/190 |

### 3.3.11 Misidentification of yeast isolates

For the purpose of this study misidentification was defined as a result which differed to that of the ITS rRNA sequence result. Misidentification was not observed using MALDI-TOF MS. Table 3.4 represents yeast misidentification observed for 5.8% (11/190) of yeast isolates using biochemical methods. 6 misidentified isolates were successfully identified by MALDI-TOF MS and 5 isolates returned a result of 'no identification', as shown in table 3.4 Misidentifications were categorised in to two groups. Major misidentification occurred for 4 isolates, where the identification given by biochemical methods has no taxonomic relatedness to that of the ITS rRNA identification. Minor misidentification occurred for 7 isolates; where the species ITS rRNA identification represents a sub-species within the clade identified by biochemical methods, table 3.4.

**Table 3.4 Results for eleven isolates correctly identified or not identified by MALDI-TOF MS and misidentified by biochemical analysis compared with ITS rRNA analysis**

| Biochemical | MALDI-TOF –MS | ITS rRNA | *n* | Class |
|---|---|---|---|---|
| *Candida krusei* | *Pichia fermentans* | *Pichia fermentans* | 2 | **Major** |
| *Candida krusei* | *Pichia cactophila* | *Pichia cactophila* | 1 | |
| *Candida pelliculosa* | No ID | *Cyberlindnera fabianii* | 1 | |
| *Candida famata* | No ID | *Debaryomyces nepalensis* | 1 | |
| *Candida glabrata* | *Candida nivariensis* | *Candida nivariensis* | 1 | **Minor** |
| *Candida parapsilosis* | *Candida orthopsilosis* | *Candida orthopsilosis* | 2 | |
| *Candida guilliermondii* | No ID | *Candida fermentati* | 3 | |

*Level of clinical impact assigned to the disagreement between the identification methods

### 3.3.12 Prospective yeast identification

Over a 12 months period MALDI-TOF MS successfully identified 99.7% (296/297) of the isolates from culture. Table 3.5 details the species distribution of the 296 isolates identified by MALDI-TOF MS. A single (n=1) isolate did not identify by MALDI-TOF MS despite obtaining excellent quality spectra. This isolate was identified as *Cyberlindnera fabianii* by ITS rRNA sequence analysis.

**Table 3.5 Prospective identification of 296 clinical yeast isolates by MALDI-TOF MS and Biotyper version 3.1**

| Identification | % | *n* | Identification | % | *n* |
|---|---|---|---|---|---|
| *Candida albicans* | 48.1 | 143 | *Candida orthopsilosis* | 0.7 | 2 |
| *Candida glabrata* | 21.2 | 63 | *Candida krusei* | 0.7 | 2 |
| *Candida parapsilosis* | 9.4 | 28 | *Candida lusitaniae* | 0.3 | 1 |
| *Candida tropicalis* | 8.1 | 24 | *Candida lipolytica* | 0.3 | 1 |
| *Saccharomyces cerevisiae* | 3.0 | 9 | *Candida nivariensis* | 0.3 | 1 |
| *Candida guilliermondii* | 1.7 | 5 | *Candida inconspicua* | 0.3 | 1 |
| *Candida kefyr* | 1.7 | 5 | *Candida pelliculosa* | 0.3 | 1 |
| *Cryptococcus neoformans* | 1.3 | 4 | *Trichosporon asahii* | 0.3 | 1 |
| *Candida dubliniensis* | 1.3 | 4 | *Candida famata* | 0.3 | 1 |

## 3.4 Discussion

This study has demonstrated that MALDI-TOF MS in conjunction with Bruker Biotyper v3.1 software is a highly accurate method for the identification of clinical yeast isolates identifying 97.4% (185/190) of yeast isolates compared with the gold standard of ITS rRNA sequencing. This is comparable to identification rates of 92 - 97.7% published in other similar studies (Dhiman *et al.*, 2011; Rosenvinge *et al.*, 2013; Sendid *et al.*, 2013). When identifying yeasts prospectively in a real-time diagnostic laboratory scenario the identification rate was improved to 99.7% (296/297) as the impact of selecting out rarer yeast species was not observed. This demonstrates that in the diagnostic setting, where the unidentified yeast species are rarely encountered, almost all clinically significant yeasts are identified by MALDI-TOF MS. The agreement between identifications achieved in this study using MALDI-TOF and ITS rRNA sequencing is due to both methods targeting the ribosomal protein components of the yeast cells for identification, similar agreement between these two methods has been reported in other studies, with Sendid *et al* reporting 97.4% (1179/1208) agreement (Seyfarth *et al.*, 2012; Sendid *et al.*, 2013). Yeast misidentification was not observed using MALDI-TOF MS in our study and has not previously been reported when utilising the manufacturer's recommended thresholds (Cassagne *et al.*, 2012; Seyfarth *et al.*, 2012; Rosenvinge *et al.*, 2013; Sendid *et al.*, 2013)

Yeast misidentification was observed using biochemical methods for 5.8% (11/190) isolates during the study. This is an important factor for diagnostic microbiology laboratories to consider. Rarely encountered yeast species have similar biochemical profiles to commonly encountered yeasts leading to misidentification. In this study the identifications obtained for 4 isolates were considered to be major misidentifications. In one example *C. lambica* was misidentified as *C. krusei*. This misidentification has previously been described in a fungaemia case report and is clinically relevant as the two species have different susceptibility patterns, particularly to the azole class of antifungal agents (Vervaeke *et al.*, 2008). Minor misidentifications occurred for 7 isolates in that the species identified by ITS rRNA sequencing were cryptic species contained within the clade identified by biochemical methods. MALDI-TOF MS was able to correctly identify 4 of these isolates demonstrating that MALDI-TOF MS has the resolution power to identify cryptic species within clades. The limitation of MALDI-TOF MS highlighted in this study was that not all species were represented in the Biotyper v3.1 database. Three *C. fermentati* isolates, belonging to the *C. guilliermondii* clade, were not identified by MALDI-TOF MS despite obtaining high quality spectra.

The Biotyper v3.1 database contains >100 yeast species within its database but is not yet fully comprehensive. Obtaining identification by MALDI-TOF MS is dependent on highly robust reference spectra being present within the database (Carrasco *et al.*, 2016). It is also possible that the database may not contain reference spectra for all strains of a species contributing to low identification log similarity scores. This was highlighted in a recent study investigating MALDI-TOF specifically for the identification of only rarely encountered yeast species (Dhiman *et al.*, 2011). An overall success rate of 84.5% was reported, with authors concluding that a result of 'no identification' indicated the yeast species was not contained within the MALDI-TOF database (Dhiman *et al.*, 2011). If MALDI-TOF MS fails to identify an organism biochemical methods must not be utilised and isolates should be referred for molecular identification.

This study has also demonstrated reducing the species log similarity score threshold alongside on plate formic acid extraction can decrease turnaround time to results for a significant proportion of yeasts in the diagnostic setting, as seen for 63.7% (121/190) of isolates in this study using a log similarity score of >1.9. Yeast misidentification was not observed as a result of reducing the log similarity score threshold for species identification. Similar approaches have been utilised in three recent studies using 70% FA on-plate extraction and a species log similarity score threshold of ≥1.7 (Cassagne *et al.*, 2012; Theel *et al.*, 2012; Van Herendael *et al.*, 2012). In two of the studies the

mean log similarity score thresholds for on-plate FA extraction (1.78 and 1.941) were significantly lower than those obtained using conventional FA extraction (2.0 and 2.223) but all log similarity score values associated with misidentification by MALDI-TOF MS were below 1.7 (Cassagne *et al.*, 2012; Theel *et al.*, 2012). If utilising on-plate FA extraction it is necessary to reduce the species log similarity score threshold to increase the identification success rate (Bader, 2013; Posteraro *et al.*, 2013). Within our local diagnostic service the species log similarity score threshold was set at >1.9 when using on plate formic acid extraction as statistically this was shown to be the most significant improvement in the identification rate. When using conventional formic acid extraction the most significant impact on the identification rate was seen by reducing the log similarity score threshold further to >1.8 with 96.3 (183/190) of the isolates identifying accurately on first attempt. A recent inter laboratory comparison study involving 11 diagnostic centres and 5460 MALDI-TOF spectra obtained using a yeast test panel demonstrated 88.6% of isolates identifying using a log similarity score of >1.7 for acceptable species identification with the conventional formic acid extraction method (Vlek *et al.*, 2014). The difference between the performance of on-plate formic acid extraction and conventional extraction can be explained through the methods used to compose the reference spectral database.

The Biotyper v3.0 reference database was developed using conventional FA extraction. The lower log similarity scores obtained using on-plate FA extraction is a result of the reduction in peak frequency and intensity compared with conventional FA extraction (Bader, 2013). The overall effect is a reduced log similarity score as fewer peaks are matched between the analyte and reference spectra. In this study 36.3% (69/190) isolates failed to identify to species level with on-plate FA extraction despite obtaining spectra. In two studies using the Vitek MS system success rates of 95.8% and 96.1% were achieved for yeast identification using the manufacturers recommended 25% FA on-plate extraction method (Iriart *et al.*, 2012; Westblade *et al.*, 2013). This can be explained by the fact that the Vitek MS database is built from reference spectra obtained using the same 25% FA on-plate extraction procedure therefore there is improved consistency between MS spectra obtained from clinical isolates and those contained within the Vitek MS reference database (Iriart *et al.*, 2012; Westblade *et al.*, 2013). This is also supported by a recent study analysing 4232 yeast isolates against an in-house database created using a unique fast sample preparation method (De Carolis *et al.*, 2012). In this study 99.5% of the isolates were correctly identified on first attempt owing to the fact that the database was constructed from reference spectra created using the same sample preparation technique used to

identify unknown clinical isolates (Elena De Carolis *et al.*, 2012). When the sample processing method is consistent between the unknown test isolate and isolate used to create reference spectra the performance MALDI-TOF systems is enhanced (Jamal *et al.*, 2014)

Based on data from this study our centre routinely tests yeast smears prepared from primary culture using on-plate FA extraction, and conventional FA extraction is performed on isolates failing to identify. The overall workflow for yeast identification in our laboratory is indicated in figure 3.3. Molecular identification is infrequently required and in this study was only required for 2.6% (5/190) of the isolates included in the validation and 0.3% (1/297) of isolates encountered in our routine practice over a 12 month period, but is the most appropriate option when MALDI-TOF MS fails to identify yeast. Molecular identification confirmed that the original identifications obtained using biochemical profiling were incorrect (unpublished data). This is a recognized limitation when using biochemical identification methods to identify rare yeast species (Bader, 2013; Posteraro *et al.*, 2013). One example, a *Cyberlindnera fabianii* isolate, was originally identified as *C. pelliculosa* using biochemical profiling. A second *Cyberlindnera fabianii* isolate was identified by ITS rRNA sequencing during the 12 month prospective evaluation after failing to identify with MALDI-TOF MS. Historically the isolation of rare yeast species was not detected in previous years due to misidentification by biochemical methods. This is an important consideration for clinical isolates in culture collections used for biotechnology applications or as a resource for reference strains in the clinical setting. Re-analysis of isolates within a Brazilian culture collection demonstrated 15% discordance between the original biochemical identification and those obtained using MALDI-TOF MS which were subsequently confirmed by ITS sequenced based identification (Lima-neto *et al.*, 2014). Using MALDI-TOF MS the isolation of rare species is now indicated by a failure to identify despite obtaining spectra. This prompts the correct application of molecular methods. The incidence of rare yeast species implicated in infections will inevitably increase as a factor of the increased accuracy of MALDI-TOF. The introduction of MALDI-TOF into the diagnostic setting has the potential to contribute to improvement in healthcare economic models and ultimately healthcare savings. The consumables cost of biochemical testing in the UK is £3.80 per sample (based on manufacturers costs within our centre). The estimated cost of MALDI-TOF is £0.50 per isolate for conventional formic acid extraction and would be less for on plate formic acid extraction (Dhiman *et al.*, 2011). A recent cost evaluation of MALDI-TOF MS for the identification of bacterial and yeast isolates reported 87.8% reagent costs savings equivalent to just

under £54,176 in one year at a large university hospital in the USA. When total costs were calculated to include technologist's time this reduced to a 51.7% cost saving of just over £56,498 (Tran *et al.*, 2015). As the turn-around time (TAT) for yeast identification is reduced from 48-72 hours using biochemical methods to under 15 minutes using conventional formic acid extraction and MALDI-TOF MS from a primary culture this will contribute to an overall cost saving for the service (Verweij *et al.*, 1999).

In summary, MALDI-TOF MS is a highly accurate method for the identification of yeasts, which may contribute to a positive impact on patient management as accurate yeast susceptibility patterns can be obtained from the identification. The increased awareness of rarer yeast species implicated in fungal infection strengthens epidemiological understanding. Furthermore maintaining a streamlined diagnostic service is essential in a modern microbiology service. Any modification that can maintain diagnostic accuracy and reduce sample-processing time has operational benefits and the addition of an on-plate formic acid extraction reference spectral database would further improve and enhance this process. This study has resulted in the discontinuation of conventional methods in our diagnostic service setting, with the introduction of MALDI-TOF as the new conventional method for yeast identification.

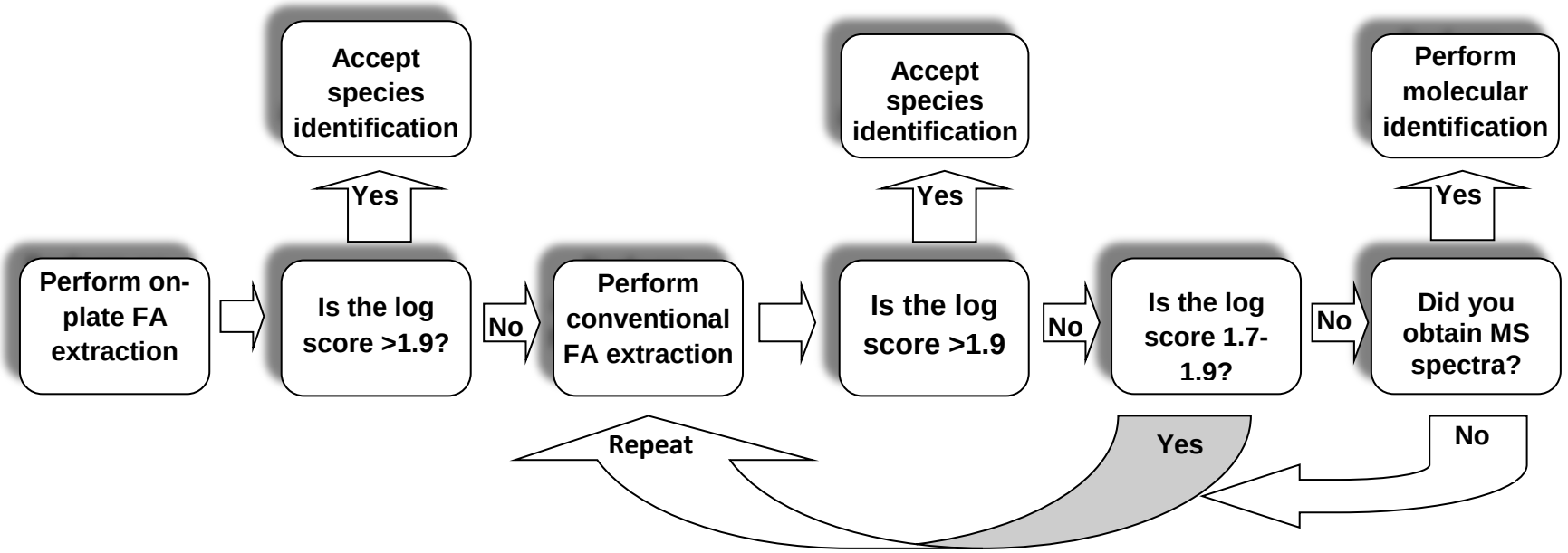



Figure 3.3 Diagrammatic representation of the yeast identification workflow in our laboratory.

# Chapter 4 Improving the diagnosis of Candidemia through the application of MALDI-TOF MS for the identification of yeasts direct from positive blood cultures

## 4.1 Introduction

In the previous chapter MALDI-TOF MS was demonstrated to be an accurate method for the identification of clinically significant yeasts from solid agar culture, Solid agar cultures typically contain upwards of $10^{10}$ cells per colony therefore, have an abundance of proteins measurable by the MALDI-TOF process. This is crucial as MALDI-TOF is reliant on a concentration greater than $10^5$ cfu/mL of biomass being present for successful identification (Lay, 2001). Identification of microorganisms direct from clinical specimens is restrictive as specimens often contain less than $10^5$ cfu/mL. Blood cultures are the exception as, when positive, they contain greater than $10^6$ colony forming units per millimetre of blood (Christner *et al.*, 2010). This makes the identification of yeasts direct from blood culture a feasible approach when using MALDI-TOF MS as cultures may contain high enough concentrations of proteins to enable detection and identification. Using MALDI-TOF MS direct on blood cultures would reduce the time to identification of yeasts by 18-24 hours, as yeasts no longer require sub-culture onto solid media and subsequent incubation prior to MALDI-TOF MS analysis. However, identification of pathogens direct from clinical specimens presents some challenges.

MALDI-TOF MS is a non-specific technique that detects all protein signatures present in an analyte regardless of origin. When analysing clinical specimens' abundant human proteins present in the sample may interfere with the analysis. The removal of human cellular components through pre-processing of the specimen is recommended prior to MALDI-TOF MS analysis to maximise the successful detection and identification of microorganisms (Bruker protocols). Specific to blood cultures, removal of erythrocytes and other blood components is necessary (Morgenthaler and Kostrzewa, 2015). Prior to this study the processing of blood cultures for MALDI-TOF MS analysis was conducted using a variety of in-house sample preparation techniques. The most frequently utilised approach is a lysis/centrifugation method. In brief, preferential lysis of erythrocytes and leucocytes occurs with reagents such as saponin, followed by washing and centrifugation to obtain pellet containing cellular debris and intact microorganisms. At the point of undertaking this

study the identification of microorganisms directly from blood culture had been performed with varied success rates ranging between 40-90% using in-house lysis/centrifugation techniques (Christner *et al.*, 2010; Ferreira *et al.*, 2010; Moussaoui *et al.*, 2010; Stevenson *et al.*, 2010; Romero-Gómez and Mingorance, 2011). Only one study chose to investigate yeast identification specifically, reporting only 5.6% (1/19) success compared with 60% overall success for bacterial isolates (Ferreira *et al.*, 2010). Furthermore, from centre to centre in-house developed methods were not standardised, with varying volumes, centrifugation speeds and reagents used. In early 2010, the Sepsityper kit (Bruker Daltonics, Germany) was launched into the diagnostic setting (Bruker Daltonics). The kit was developed for the pre-processing of blood cultures and consisted of two buffers, the first a red cell lysis buffer and the second a detergent wash buffer. Through centrifugation and treatment with the buffers the process was designed to remove the blood fraction of the culture leaving behind a pellet of concentrated microorganisms that could then be carried forward for MALDI-TOF MS analysis. The processing time was claimed to less than 10 minutes for a single blood culture, making this a rapid method for identifying organisms direct from blood cultures. The aim of this study was to evaluate and optimise blood culture processing using Sepsityper (Bruker Daltonics, Germany) specifically for yeast identification from blood cultures to reduce the time to diagnosis of candidemia.

This study first focused on optimisation of the blood culture processing method using the Sepsityper assay (Bruker Daltonics, Germany) with several modifications to the method accounting for the change in microorganism detection. Once optimised, the blood culture processing method was applied to a larger set of yeast blood cultures to evaluate performance.

## 4.2 Methods

### 4.2.1 Yeast isolates

The optimisation arm of the study contained ten yeast isolates from confirmed cases of fungaemia, as detailed in table 4.1. For the final method of evaluation, fifty yeast isolates were selected from proven cases of fungaemia. Isolates were stored and passaged as detailed in general methods 2.1.

### 4.2.2 Conventional phenotypic identification

See general methods 2.1

### 4.2.3 Formic acid extraction of proteins from solid agar yeast cultures

See general methods 2.3

### 4.2.4 Simulated blood cultures

See general methods 2.1

### 4.2.5 Miles and Misra cfu/mL counts

See general methods 2.1

### 4.2.6 Standard Sepsityper blood culture processing

200µL Sepsityper lysis solution (Bruker Daltonics, Germany) was added to a 1mL aliquot of blood and vortexed for 10 seconds. After incubation for 2-3 minutes at room temperature the suspension was centrifuged at 8000g for 2 minutes. The supernatant was discarded (avoiding disturbance of pellet) and the pellet re-suspended in 1mL of Sepsityper washing solution (Bruker Daltonics, Germany) by vortexing for 30 seconds. Centrifugation was repeated at 8000g for two minutes and the supernatant discarded. The pellet was re-suspended into 300µL of water by vortexing and 900µL absolute ethanol was added, vortexed, and a centrifuged again at 8000g for two minutes. The supernatant was discarded completely and the pellet was air dried for 20 minutes. 30µL of 70% formic acid was added to the pellet, vigorously mixed (including washing the acid along the side of the tube repeatedly to reconstitute any adhered cellular material) pulse centrifuged, pellet re-suspended, then incubated for 3 minutes. To this 30µL of acetonitrile was added, mixed well, and after a second centrifugation at 8000g for two minutes, the final supernatant was used for analysis.

### 4.2.7 Optimisation of Sepsityper blood culture processing

Several variables were identified within the bacterial Sepsityper assay procedure, listed below as experiments a) – f). Figure 4.1 illustrates the stage at which each variable was evaluated within procedure. If a modification was found to be beneficial to the yeast assay development it was retained within the method in a cumulative approach.

a) Increasing blood volume to 5mL

b) Increasing 100% Formic acid volume from 30µL to 70µL.

c) Additional pellet wash with solution 2

d) Water pre-wash; 1 mL of the blood culture fluid was centrifuged at 8000g for 2 minutes, and the cell pellet was washed twice with 1mL of water

e) Glass bead disruption of pellet; approximately 50µL equivalent volume of glass beads added to the cell pellet with the formic acid and vortexed for 5 minutes

f) Vortexing the blood culture; gentle vortexing of the BacTec bottle at 1400rpm for 30 seconds. Further vortexing of the 1ml blood aliquot for five minutes at 1400rpm prior to processing**.**

### 4.2.8 Final optimised Sepsityper blood culture processing method

All centrifugation steps were performed for 2 minutes at 8000g**.** Blood culture bottles were vortexed gently for 30 seconds, 1mL aspirated and transferred to a Sepsityper tube and vortexed for 5 minutes. After vortexing**,** 200µL of Sepsityper lysis buffer (LB) was added, vortexed for 10 seconds and incubated for 2 minutes. The suspension was centrifuged, supernatant discarded (avoiding disturbance of pellet) and the pellet re-suspended in 1mL of Sepsityper washing buffer (WB). Centrifugation was repeated, the supernatant discarded and the pellet was re-suspended into 300µL water and 900µL of absolute ethanol and re-centrfuged as previous. The supernatant was discarded and the pellet air-dried for 5 minutes at room temperature. If a blood culture analysis did not return the identification on first attempt repeat analyses was undertaken

### 4.2.9 Formic acid extraction of blood culture pellets

70µL of 70% formic acid was added to the dry pellet. The pellet was completely re-suspended by pipetting (washing the formic acid along the side of the tube) and incubated for 5 minutes at room temperature. An equal volume of acetonitrile was added, mixed well, and then centrifuged. The target plate was inoculated with 1µL of the supernatant in triplicates and once dried, immediately overlaid with 1µL α-Cyano-4-hydroxy-cinnamic-acid matrix.

### 4.2.10 MALDI-TOF spectral analysis

See general methods 2.6 for standard identification protocol. For the amended blood culture analysis Spectra were cropped to exclude the mass range 0-4000 kDa and log similarity scores were lowered to >1.6 for genus identification and >1.8 for species identification. In a second analysis ‘adjusted log similarity score parameters’ were investigated to accept all identifications at species level with log similarity scores >1.5 providing the top three matches were the same species.

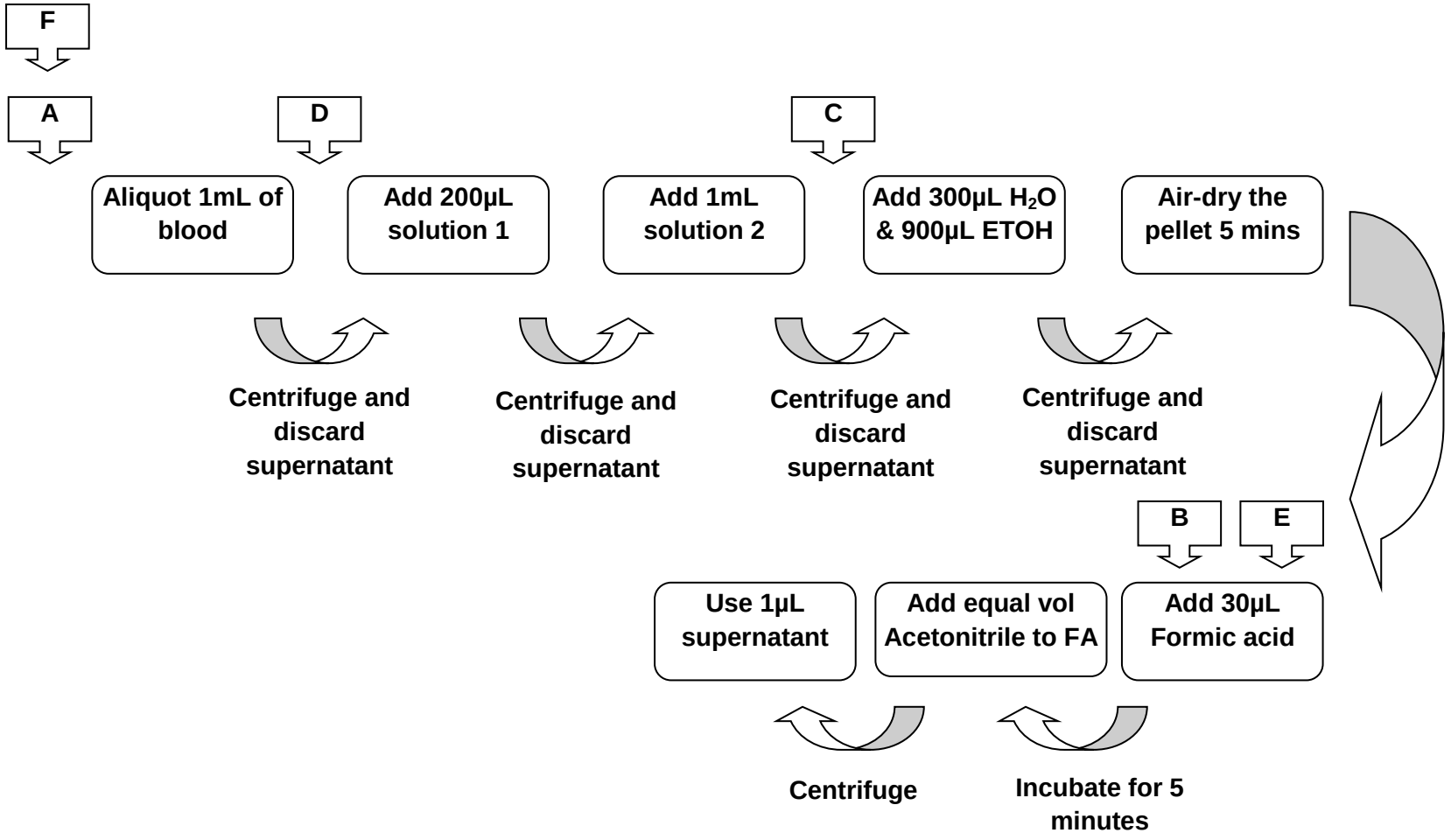



Figure 4.1 Diagrammatic representation of Sepsityper processing with modifications indicated from A-F at the point of the process where they were introduced; a) increased blood volume, b) formic acid volume increase to 70µL c) additional wash solution 2 step d) water pre wash e) glass bead disruption of the pellet f) vortexing of the blood culture bottle and 1mL aliquot.

## 4.3 Results

### 4.3.1 Yeast identification from CHROMagar® *Candida* cultures

ITS rRNA analysis from CHROMagar cultures returned the following identifications; *C. albicans* (17/50), *C. glabrata* (9/50), *C. tropicalis* (4/50), *C. krusei* (4/50), *C. parapsilosis* (7/50), *C. orthopsilosis* (1/50), *C. metapsilosis* (1/50), *C. guilliermondii* (2/50), *C. lusitaniae* (1/50), *Cryptococcus neoformans* (2/50), *Magnusiomyces capitatus* (1/50), *Cyberlindnera fabianii* (1/50). MALDI-TOF MS analysis identified 98% (49/50) of yeast isolates from CHROMagar culture. A *Cyberlindnera fabianii* isolate was not identified by MALDI-TOF MS, table 4.1.

Table 4.1 Yeast identifications by MALDI-TOF and ITS rRNA sequencing from CHROMagar® *Candida* cultures

| **MALDI-TOF** | **%** | ***n*** | **ITS 1&2** | **%** | ***n*** |
|---|---|---|---|---|---|
| *C. albicans* | 34 | 17 (3) | *C. albicans* | 34 | 17 |
| *C. tropicalis* | 8 | 4 (2) | *C. tropicalis* | 8 | 4 |
| *C. glabrata* | 18 | 9 (1) | *C. glabrata* | 18 | 9 |
| *C. krusei* | 8 | 4 | *C. krusei* | 8 | 4 |
| *C. parapsilosis* | 14 | 7 (1) | *C. parapsilosis* | 14 | 7 |
| *C. orthopsilosis* | 2 | 1 | *C. orthopsilosis* | 2 | 1 |
| *C. metapsilosis* | 2 | 1 | *C. metapsilosis* | 2 | 1 |
| *C. guilliermondii* | 4 | 2 (1) | *C. guilliermondii* | 4 | 2 |
| *C. lusitaniae* | 2 | 1 | *C. lusitaniae* | 2 | 1 |
| *C. neoformans* | 4 | 2 (1) | *C. neoformans* | 4 | 2 |
| *M. capitatus* | 2 | 1 (1) | *M. capitatus* | 2 | 1 |
| *NO ID* | 2 | 1 | *Cyberlindnera fabianii* | 2 | 1 |
| **Total** | **100** | **50** | | **100** | **50** |

() Brackets indicate the isolates used in the optimisation arm of the study

### 4.3.2 Optimisation of Sepsityper blood culture processing

Miles and Misra counts demonstrated 100% (10/10) of the blood cultures used for method optimisation flagged positive > $10^6$ cfu/mL.

Using the standard Sepsityper methodology 20% (2/10) of the blood cultures were identified at genus level including one *C. albicans* isolate (log similarity score 1.942) and one *C. parapsilosis* isolate (log similarity score 1.93). Increasing blood volume led to complete failure of detection across all blood cultures with no yeasts being identified.

Increasing the volume of formic acid applied to the pellet (70µL) during protein extraction increased the success rate to 10% (1/10) for species identification of one *C. albicans* (log similarity score 2.08), and a genus identification success rate of 50% (6/10) with four further isolates being identified including *C. tropicalis* (2) (log similarity scores 1.936, and 1.866), *M. capitatus* (log similarity score 1.971) and a second *C. albicans* isolate (log similarity score 1.823).

The positive impact of increased FA volume is demonstrated in figure 4.2 with a significant shift (p=0.02) in the median log similarity score obtained across the ten analyses. The addition of a second wash step using Sepsityper wash solution 2 did not significantly increase the success rate further nor did the addition of a water pre-wash step, which had a negative impact reducing the overall success rate to 50% (5/10). No improvement was seen with the addition of vortexing with glass beads to the formic acid incubation stage. Vortexing of the blood culture bottle and 1mL aliquot prior to processing increased the overall success rate to 70% (7/10) and also improved the species identification rate from 10% (1/10) to 40% (4/10) for those yeasts detected from the blood cultures.

### 4.3.3 Modification of MALDI-TOF MS spectra prior to identification

Re-analysis of the mass spectra obtained using the optimised Sepsityper™ method was performed using the amended blood culture spectral analysis protocol that cropped spectra at 4000kda. The overall identification success rate doubled to 80% (8/10) with 60% (6/10) identified to the species level (log similarity score >2.0) and 20% (2/10) blood cultures identified to the genus level (log similarity score >1.8). Figure 4.2 and table 4.2 demonstrate the significant shift in the median log similarity score after adjustment of spectral analysis. By lowering the acceptable log similarity scores for identification to >1.8 as per the blood culture spectral analysis protocol all eight yeasts were now classified at the species level. Two yeasts remained unidentified from blood culture, one *C. neoformans* isolate and one *C. glabrata* isolate.

Whilst vortexing the blood did not significantly impact the median log similarity score value it contributed to two additional blood cultures being identified with a log similarity score >2.0 so was retained as a modification in the method. No other modification had a statistically significant impact on the log similarity scores obtained, as shown in table 4.2 and figure 4.2.

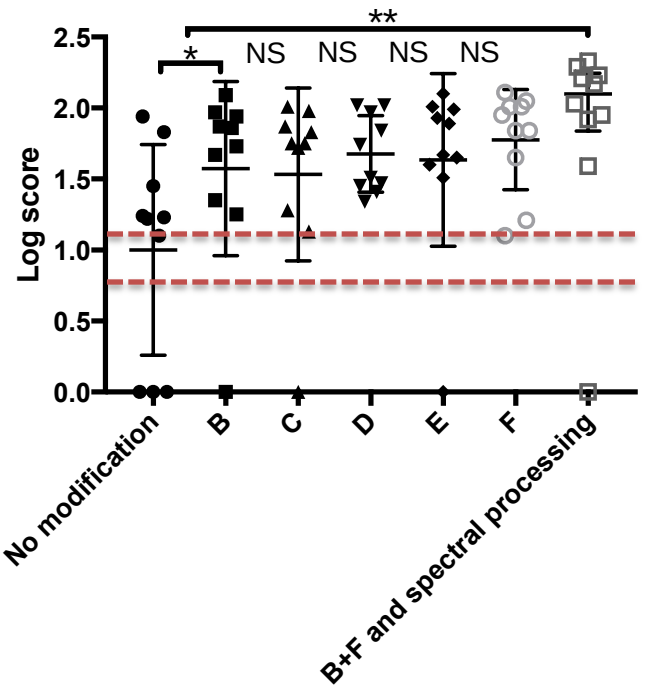



Figure 4.2 Scatter plot of average log similarity scores obtained from triplicate analyses of ten yeast blood cultures processed by Sepsityper with the addition of modifications: B = Increased formic acid to 70µL, C = Extra solution 2 wash, D = Pre water wash, E = Bead beating pellet, F = vortexing blood culture bottle and 1mL aspirate. Adjusted spectral analysis included cropping out the 0-4000kDa range from MS spectra. **p<0.01, *p=0.02, NS = no significant difference. Red lines indicate thresholds of >1.7 for genus identification and >2.0 for species identification.

**Table 4.2. Median log similarity scores obtained through modifications to the Sepsityper processing method**

| **Modification to Sepsityper processing** | | **Log similarity score** | | | **Significance** |
|---|---|---|---|---|---|
| | | Median | IQR | | |
| A | No modification | 0 | 1.23 | 1.55 | - |
| B | Increased volume formic acid | 1.8 | 1.33 | 1.95 | *P=0.02 |
| B+C | Second buffer 2 wash | 1.75 | 1.24 | 1.9 | 0.84 |
| B+D | Water pre wash | 1.63 | 1.44 | 1.98 | 0.78 |
| B+E | Glass bead beating | 1.78 | 1.58 | 2.0 | 0.49 |
| B+F | Vortexing | 1.9 | 1.54 | 2.0 | 0.54 |
| 70µL formic acid, vortexing with spectral processing | | 2.1 | 1.84 | 2.245 | **P<0.01 |

### 4.3.4 Application of the final optimised blood culture processing method and MALDI-TOF MS spectral analysis protocol to blood cultures

The optimised blood culture processing method was applied to a set of 50 blood cultures to evaluate its performance. MALDI-TOF MS identified 56% (28/50) of the yeasts from blood cultures at the species level on first attempt. Of the remaining blood cultures, 20% (10/50) returned a genus only identification of *Candida* sp with a log similarity score >1.6 but <1.8 and the remaining 24% (12/50) returned a log similarity score <1.6 with a result of 'no reliable identification'.

92% (46/50) of spiked blood cultures flagged positive with a concentration greater than $1x10^6$ cfu/mL and the remaining four cultures contained greater than $1x10^{5.}$ No correlation was seen with cfu/mL and failure to identify yeasts within blood cultures, shown in figure 4.3.

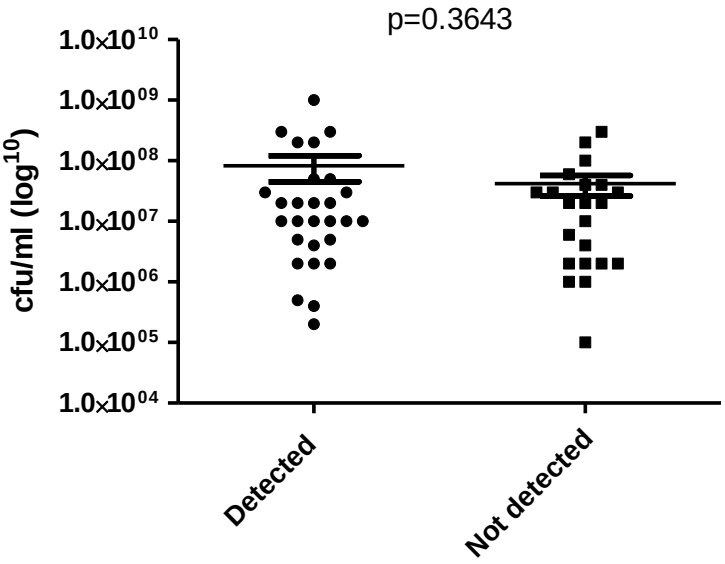



Figure 4.3 Graph plotting cfu/mL concentrations for 50 blood cultures included in the blood culture processing evaluation, grouped into those blood cultures which yeasts were detected from versus those that yeasts were not detected from. No significant difference was observed between the two groups (p=0.36).

Re-analysis of MALDI-TOF spectra using ‘adjusted’ parameters (including the lowered species log similarity score threshold of >1.5) for the 20% (10/50) of the blood culture strains identified to genus level, re-classified these results to acceptable species identification with no observed misidentification, increasing the overall success rate to 76% (38/50).

Re-analysis of spectra for the 24% (12/50) of blood cultures with a log similarity score <1.5 with adjusted parameters had no effect. Repeat processing of these blood cultures was performed and 8% (4/50) were resolved, all returning a correct identification. 16% (8/50) of yeast from blood cultures still remained undetected. Figure 4.4 demonstrates spectra from a *C. albicans* blood culture where yeasts failed to identify (B) alongside a spectrum from a *C. albicans* blood culture where yeasts were successfully identified (A) demonstrating a lack of intensity for peaks obtained from yeast proteins. No correlation was observed between yeast species and unsuccessful identification.

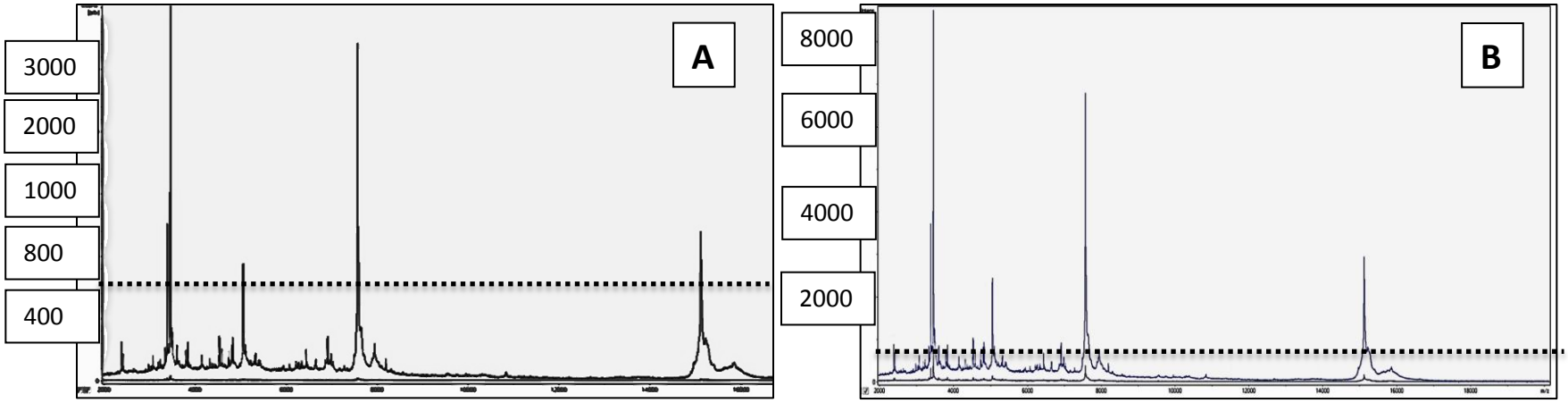
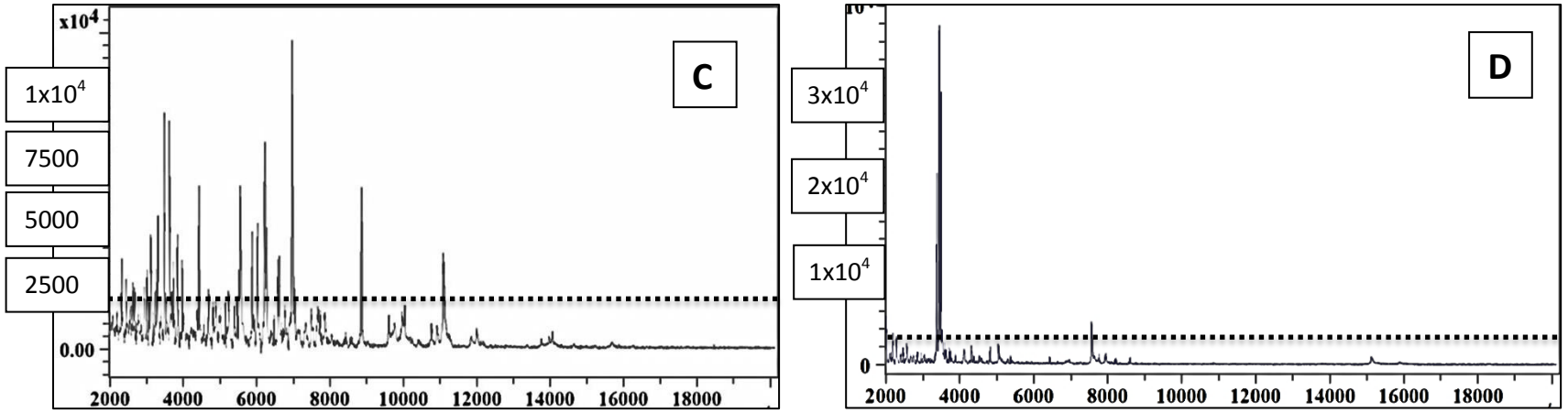



Figure 4.4 Sections of MALDI-TOF MS spectra at acquisition for a) *Candida albicans* blood culture analysis with a log similarity <1.6 indicating 'no identification (b) *C. albicans* blood culture with a successful identification with a log similarity score >1.8 (c) a *Candida albicans* isolate identified by MALDI-TOF log similarity score 2.312 (d) spectrum obtained from a negative blood culture by MALDI-TOF MS. The dotted line on both spectra indicates 600 absolute intensity.



## 4.4 Discussion

This study has demonstrated that MALDI-TOF MS is capable of identifying yeasts direct from blood cultures, with 76% (38/50) accuracy compared with MALDI-TOF MS identification from solid agar. This success rate was achieved after optimisation of blood culture processing and spectral analysis. In a recent meta-analysis of Sepsityper performance, this success rate is supported by a recent meta analysis including 21 studies which reported a success of 66% for MALDI-TOF MS yeast identification from blood cultures (Nils G Morgenthaler and Kostrzewa, 2015). The authors acknowledged the challenges faced when identifying yeasts specifically from blood cultures. Prior to optimisation Sepsityper processing was completely unsuccessful for the identification of yeasts at species level assuming a log similarity score of >2.0, despite blood cultures having a cfu/mL concentration $>10^5$, which is considered sufficient for MALDI-TOF MS detection (Yan *et al.*, 2011). In a recent study Yan *et al* reported complete failure to identify yeast with direct application of Sepsityper to yeast blood cultures. In a similar study, utilising an in house lysis centrifugation-processing method, Garci *et al* ( 2010) demonstrated poor performance identifying only 5.6% (1/19) yeasts from blood cultures by MALDI-TOF MS (Garcı, 2010), with other studies also demonstrating poorer performance for yeasts (Chien *et al.*, 2016; Jeddi *et al.*, 2016) This indicates strongly that the standard methods used for bacteria are not appropriate for yeast identification from blood culture.

### 4.4.1 Optimisation of blood culture processing and spectral analysis

Failure to detect yeasts by MALDI-TOF MS is associated with two factors. The intensity of the peak represented in the MS spectra for yeast proteins are far less than the intensity seen with bacterial analysis. This in part is due to the robust cell wall of the yeast cells resisting lysis by formic acid. The second factor is the carryover of unwanted proteins from blood during processing. Three clusters of high intensity peaks, common across all analyses are visible within blood culture spectra (Figure 3.7 D). The Sepsityper assay is designed to remove cellular blood components by lysis. However, carry-over free blood proteins are not removed by the processing. This study investigated a variety of modifications to blood processing with the aim of optimising Sepsityper processing for yeast blood cultures. Additional steps found to be beneficial to the success of identification included: increased volume of formic acid and subsequently the volume of acetonitrile applied to the pellet, vortexing the blood

culture, adjustment of spectral analysis with cropping from 0-4000 kDa and finally a reduction in the log similarity scores thresholds applied to the level of identification.

### 4.4.2 Modification to blood culture processing

#### 4.4.2.1 Formic acid volume

The volume of 100% formic acid applied to the yeast pellets varies across studies utilising the Sepsityper assay between 2µL to 70µL (Haigh *et al.*, 2011; Jamal, Saleem and Rotimi, 2013; Nonnemann, Tvede and Bjarnsholt, 2013; Tadros and Petrich, 2013; Martinez *et al.*, 2014). In the majority of studies the volume was determined by pellet size. In an earlier study 30µL of FA was added to a pellet obtained from 8mL of blood (Spanu *et al.*, 2012) and an overall success rate of 91.8% was achieved, although in this study identifications were accepted with a log similarity score >1.2 providing all matches were the same species. Conversely in this study 70µL of formic acid was found to be more successful when applied to a pellet obtained from a blood culture volume of 1mL, increasing the success rate from 20% (2/10) to 60% (6/10). The reasons for the conflicting observations are not clear. Improved detection when increasing the volume of FA in this study may have been due to a dilution effect on blood proteins allowing yeast proteins to be detected or may have contributed to more efficient lysis of the yeast cells.

#### 4.4.2.2 Vortexing

The addition of vortexing steps both for the blood culture prior to aspiration and the aspirated 1mL aliquot slightly improved the overall success rate but most notably had a positive impact on identification at the species level with two additional blood cultures achieving a log similarity score >2.0. This is the only study to describe vortexing as a modification to optimise the success of Sepsityper. Morphology of yeasts in blood culture may be an important factor. Hyphae and blastospores aggregate in blood cultures. By vortexing the blood culture it may liberate blastospores and hyphae into a unicellular state, allowing for a more consistent distribution of yeasts when aspirating the blood culture from the bottle and may also increase the surface area accessible by the FA, allowing for more efficient extraction of proteins.

No other modifications to the blood culture processing were found to be beneficial in this study, although it is acknowledged that we used a small sample size of ten blood

cultures. Increasing the blood culture volume processed to 2mL resulted in complete failure of detection, most likely due to an increase in the human protein component of the analyte. By doubling the blood culture volume the amount of human protein extracted through the process is doubled in concentration. The yeast protein peaks were not visible in the MALDI-TOF MS spectra. As all blood cultures were found to contain a concentration of organisms within the published limit of detection (LOD) for MALDI-TOF MS analysis we did not consider it necessary to increase the blood volume any further (Drancourt *et al.*, 2016) as this would have also required excessive reagents from the Sepsityper kit. It is important to acknowledge that increased blood volume may have contributed to the increased success rates observed in two similar studies. Ferroni *et al* demonstrated a success rate of 95.2% from 21 blood cultures using 1.8mL of blood (Ferroni *et al.*, 2010). In a recent report a success rate of 91.8% was achieved with 8mL of blood culture (Spanu *et al.*, 2012) however, using almost exactly the same processing method and 8mL of blood a second study reported a poor success rate of 9.3% (5/54) for the identification of yeasts (Rodríguez-Sánchez *et al.*, 2013). Aspirating 8mL of blood for centrifugation has considerable health and safety considerations and is not a desirable approach for the routine diagnostic setting.

Additional wash steps prior to lysis and a second buffer 2 wash were also investigated. Additional wash steps did not improve success any further after the increased volume of FA was added and again led to the utilisation of more Sepsityper reagents. In contrast 100% success has been observed with the addition of two wash steps prior to the red cell lysis. However, in this study the authors also used a larger than recommended volume of FA (50µL) which could have contributed to the increased success rate (Yan *et al.*, 2011). In a more recent study, an in-house approach utilising SDS as the primary lysis solution, with a secondary water wash step, was found to be superior to Sepsityper processing, improving the identification rate by 8% across 107 yeast blood cultures (Bidart *et al.*, 2015). This suggests that adjusting the reagents used to process yeast blood cultures could contribute to increased success. Despite all of this a significant proportion of yeast remains undetectable from blood cultures, due to endogenous blood proteins remaining present in the analyses. Other factors may be important during blood culture processing. Increasing the number of protein extract replicates spotted onto the target plate may increase the success rate. In this study extracts were spotted in triplicate whereas in the more successful study protein extracts were spotted in quadruplicate (Yan *et al.*, 2011). The culture medium used may also serve to increase the success of MALDI-TOF MS. Spanu *et al* utilised BACTEC Mycosis-IC/F culture vials that are optimised for fungal cultures and contain

saponin, a red blood cell lysing agent. This effectively added an extra red cell lysis step, which may have contributed to the increased success rate (Spanu *et al.*, 2012). Despite all of these modifications to blood culture processing the identification of yeasts is still negatively affected by the presence of human protein peaks in spectra.

It is important to acknowledge that any modification would deviate from using a CE-IVD regulated assay, which in a diagnostic service laboratory may have regulatory and quality control implications and would require a validation study. In-house developed methodologies require significantly more validation data to support claims for introduction into diagnostic practice. These processes can be counter intuitive and can often be costly for the laboratory to maintain analytical performance both in on-going supply of reagent and assay components and in terms of the increasing complexity of regulatory systems which govern the use of in vitro diagnostics in clinical practice systems (Morgenthaler and Kostrzewa, 2015)

### 4.4.3 Spectral analysis and adjusted log similarity scores

The blood culture analysis program in the Biotyper software is designed to exclude the spectral range 0-4000 kDa from the identification-matching algorithm. The purpose of this is to remove the cluster of blood protein peaks with the highest intensity, which has a peak position around 3500 m/z (mass to charge ratio). This resulted in increased identification success rate detection and increased log similarity scores across analyses. The log similarity score is in part calculated from the intensity of peaks within the unknown spectra, a full explication of the log similarity score can be found in the general method section 2.3. The principle is the higher the intensity the greater the score for a peak. If a peak is matched between the unknown and reference spectra it is given a positive score, whereas unmatched peaks are given a negative score. By removing the high intensity blood protein peaks from the analysis it removes the negative impact of these unmatched peaks on the log similarity score and allows the positive scores from lower intensity yeast proteins to have more weight. Figure 4.4 demonstrates spectra acquired from two blood cultures containing *C. albicans*. In figure 4.4 (A) a result of ‘no identification’ was achieved, as the intensity of yeast proteins are a fraction of those obtained from the blood whereas. In figure 4.4 (B) successful species identification was achieved as the yeast proteins have greater intensity within the spectrum and cross the acceptable threshold for peak intensity despite the presence of highly abundant human proteins in figure 4.4 (C) highly abundant protein with strong peak intensities match those of blood proteins and the presence of the blood peaks is neutralised.

### 4.4.4 Evaluation of optimised methodology

Using the optimised blood culture processing method and adjusted spectral analysis MALDI-TOF MS demonstrated a first attempt success rate of 56% (28/50). Several recent studies have reported much higher success rates between 91.8% and 100% (Ferroni *et al.*, 2010; Yan *et al.*, 2011; Spanu *et al.*, 2012; Pulcrano *et al.*, 2013). There are several factors that may have contributed to the lower success rate of MALDI-TOF MS in this study compared with these other studies. Processing error was accounted for by repeating analysis of blood cultures when identification was not achieved, but from a workflow perspective in the diagnostic laboratory high proportions of repeat analyses would not be ideal. The success rate increased to 64% (32/50) with four further cultures achieving identification. Loss of pellet during processing was the likely factor leading to no identification in these cultures. However, 36% (18/50) cultures remained unidentified.

This study has demonstrated that applying an adjusted set of parameters, including lowering the acceptable species log similarity score threshold further to >1.5, increases the success rate to 76% (38/50) on first attempt without misidentification, and 84% (42/50) when including repeat analysis. Lowering the score thresholds was justified through observing that when yeasts were detected only the correct yeast species, compared against ITS rRNA sequencing, were returned as identifications with log similarity scores above and below the acceptable species threshold. In all instances at least the top 3 differential identifications were of the same species. This approach has also been demonstrated in a previous study with similar success rates (Schubert *et al.*, 2011). Whilst lowering log similarity scores and cropping spectra improves the success of MALDI-TOF MS identification it is evident that the quantity of protein from blood components far outweighs yeast proteins. The high concentration of human protein may have an impact during matrix co-crystallisation (Drancourt *et al.*, 2016). Highly abundant human protein could overwhelm any yeast proteins present which in turn then fail to co-crystallise. The success rate of MALDI-TOF MS for the identification of yeast from blood cultures may be improved if total human protein can be eliminated during processing prior to formic acid extraction of the yeast pellet.

The outstanding factor not investigated in our study was the blood and its associated components. The use of five-day negative blood for spiking development experiments may have impacted on the overall success rate. It is possible that older blood contains larger amounts of free human protein released during haemolysis however, as the time to a positive blood culture is reportedly up to 120 hours for some *C. glabrata* isolates

(George, Horvath and Hospenthal, 2005), ‘older’ blood will inevitably be analysed using MALDI-TOF MS for yeast identification. Furthermore the use of five-day negative blood did not affect the viability of the yeast cultures on sub-culture. As the negative impact of using five day negative blood was not universal across analyses it may be better explained by the fact that blood from different patient may have a variable impact on MALDI-TOF MS analysis. The study of specific properties within the blood is an important consideration for further work.

In summary, whilst Sepsityper alongside MALDI-TOF MS analysis is applicable in its current format for bacterial identification from blood cultures, improvement and standardisation of the processing method for Yeast identification is required to achieve the success rate required by a routine diagnostic laboratory. Methods to reduce the impact of human components within the analyte also require further refinement to progress these methods for diagnostic use.

# Chapter 5 Evaluation of MALDI TOF MS compared with conventional microscopic identification of filamentous mould isolates from clinical samples

## 5.1 Introduction

The ability to accurately identify fungi to species level using macro- and microscopic features requires skill and expertise. This comprehensive process is challenging for general microbiology laboratories that have a high diagnostic mycology workload. This is reflected in recent studies reporting the accuracy of conventional identification of filamentous fungi by microscopy to be between 61.5%-80.1% (Becker *et al.*, 2014; Gautier *et al.*, 2014; Ranque *et al.*, 2014). For non-*Aspergillus* isolates the successful identification to the species level can be as low as 31% in the diagnostic setting when compared with gold standard molecular identification (Ranque *et al.*, 2014).

There are several limitations to conventional identification that contribute to misidentification. To date over 100,000 fungi have been taxonomically classified, yet many more remain unknown. Many environmental fungi share similar macro/microscopic characteristics with pathogenic fungi when cultured under laboratory conditions; therefore misidentification of rarely encountered fungi is always a risk. The laboratory also relies on published material to aid the fungal identification process but literature can rapidly become out of date as fungal taxonomy is changing constantly through whole genome sequencing reclassifying species that were previously classified using morphological characteristics (de Hoog and Guarro, 1995; Campbell, 2013). In the laboratory environment some fungal strains fail to produce fruiting structures, making it impossible to identify them phenotypically. This instance the laboratory is reliant on molecular identification techniques, such as sequence-based analysis of rRNA regions (Borman *et al.*, 2008). Most diagnostic laboratories do not have direct access to molecular facilities; therefore isolates are referred to a reference laboratory. This process extends the turnaround time of identification. The other major consideration for most laboratories providing a comprehensive mycology service is that laboratory scientists require several months of training to become competent in fungal identification. Trained scientists need frequent rotation through the mycology laboratory to maintain their competency assessment profiles. As a result laboratories often have a single scientist who is relied upon for the provision of their mycology service. This is not a robust approach to providing a comprehensive

mycology service in modern health care environment. If Mycology is to sustain itself within pathology a new approach to fungal identification is required.

Maier and colleagues recognised this when they published the first standardised method for fungal identification using MALDI-TOF MS (Maier and Kostrzewa, 2009). The authors proposed a new method for cultivating fungi in liquid medium encouraging the growth of mycelia to form 'fungal balls' with an absence of fruiting structures and conidia. The production of high quality reproducible spectra was possible through applying a standard formic acid extraction protocol to the mycelial cultures (Maier and Kostrzewa, 2009). In 2011 a filamentous fungal v1.0 (FFv1.0) database became commercially available (Bruker Daltonics, Germany). The database contained MS spectra representing 104 species (365 spectra) as generated using the novel liquid cultivation method. Subsequently this was recommended as the processing method for fungal isolates when using the FFv1.0 database is the liquid cultivation method (Maier and Kostrzewa, 2009).

The aim of this study was to evaluate the FFv1.0 database, (Bruker Daltonics, Germany) utilising the liquid cultivation method for the identification of fungi. Gold standard ITS rRNA analysis and conventional identifications were compared to those obtained by MALDI TOF MS.

## 5.2 Materials and Methods

### 5.2.1 Sample selection

Filamentous fungi surplus to diagnostic requirements were selected from a clinical archive at the UK CMN regional mycology laboratory, Royal Free Hospital microbiology laboratory. A range of species was selected to represent a diverse collection of fungi encountered in the diagnostic setting. All fungi had previously been isolated from clinical specimens and were considered to be pathologically significant. Isolates were passaged as detailed in general methods section 2.1.2.

### 5.2.2 Microscopic identification

Microscopic identification was performed by the 'advanced' scientist experienced in fungal identification with the aid of two identification keys (de Hoog and Guarro, 1995; Campbell Colin K, 2013). Sellotape and needle mount preparations were prepared as per published methods with Lactophenol cotton blue (Campbell Colin K, 2013).

Phenotypic identification was based on macro-morphological criteria including; growth on different media and at different temperatures, colour, and growth rate of the colony, and on micro-morphological criteria, e.g. conidiogenesis, shape of conidia and conidia, and mycelial structures, (Campbell Colin K, 2013).

### 5.2.3 Culture of mycelial mats for DNA extraction

To minimise the potential for cross contamination of DNA extracts, each fungal isolate was cultured as a non-sporing filamentous 'pancake like' mycelial mat. All processing was performed in a laminar flow cabinet to protect cultures from environmental contamination. To produce the mycelial mats conidia were harvested from each fungal colony and a suspension was prepared in saline. 100µL of each conidial suspension was inoculated into a 96-well microtitre plate containing 5mL of Sabouraud's liquid media. The microtitre plates were incubated for 1-3 days under daily observation. Once a complete mycelia mat had formed, each culture was harvested onto a sterile Whatman filter paper (grade 3) and transferred to a 5mL bijou. The mycelia mats were stored at -70°C until required for DNA extraction.

### 5.2.4 DNA extraction from mycelia mats

See general methods 2.2.1

### 5.2.5 PCR amplification and sequencing of the ITS rRNA

See general methods 2.2.2 - 2.2.4

### 5.2.6 Referral of unidentified isolates to reference facilities for further molecular identification

See general methods 2.4

### 5.2.7 Cultivation of mycelia balls

Fungal culture plates were manipulated inside plastic sample bags to control the dispersion of conidia into the environment. Using a moistened cotton swab the top of the petri dish was lifted slightly and the cotton swab was gently rolled over the surface of the fungal culture to harvest conidia. The swab was immediately transferred to a 15mL falcon tube containing 10mL of Sabouraud's liquid. The conidia were released from the swab by pressing it against the side of the tube. The falcon tube was then sealed and inverted to mix. Broths were incubated for 24 hours on a SB2 rotator at 30°C.

Optimisation of the fungal inoculum was undertaken in a crude approach by inoculating a very light, light and moderate quantity of conidia into the Sabouraud's liquid broth to establish which spore inoculum warranted the best sporulation of fungal mycelium. Throughout processing a control tube of Sabouraud's liquid agar was placed in the preparation area and left open to control for contamination events.

### 5.2.8 Harvesting the mycelia balls and protein extraction

The Falcon tubes were removed from the incubating rotator and centrifuged for 10 minutes at 4000g. Using a fat tip pastette the pellet and approximately 1.5mL of SAB broth was transferred into a 1.5mL microfuge tube. The eppendorf was centrifuged at 8000g for 2 minutes. The supernatant was discarded carefully using a fine tip pastette. The pellet was re-suspended in 1mL of distilled water then centrifuging again at 8000g for 2 minutes. The supernatant was removed using a fine tip pastette. The respective water wash step was repeated once more.

### 5.2.9 Formic acid extraction

See general methods 2.3.1 with the exception of the ethanol-drying step as detailed in section 5.3.13.

### 5.2.10 Optimisation of pellet dehydration

Four fungal isolates were selected, for method optimization representing species commonly encountered: *Aspergillus fumigatus* (1), *Aspergillus oryzae* (1), *Mucor heimalis* (1) and *Fusarium oxysporum* (1). Three replicates of each isolate were prepared as fungal balls and processed in parallel using the three pellet treatment steps a) – c) below. The protein extracts were spotted in quadruplicates.

a) Pellets were placed in a hot block for 20 minutes at 60°C.
b) Pellet was left to dry in an incubator at 37°C for 2 hours.
c) No drying of the pellet.

### 5.2.11 MALDI-TOF analysis of protein extracts and identification with the Biotyper 3.1 software

See general methods 2.3.2 and 2.3.3.

## 5.3 Results

### 5.3.1 Sample selection

In total 147 clinical isolates were included in the sample cohort representing 24 genera and 47 species as determined by iTS rRNA sequenced based identification. Table 5.1 details the ITS rRNA identifications for the 147 strains. *Aspergilli* represented 49% (72/147) of the isolates with the remaining 51% (75/147) being non-*Aspergillus* species.

### 5.3.2 ITS rRNA identification of fungal isolates

In-house ITS rRNA sequenced based identification was achieved for 87.8% (129/147) fungal isolates. The remaining 12.2% (18/147), all *Aspergillus* species, were referred to Bristol Mycology reference laboratory for molecular identification as per published protocol (Borman *et al.*, 2010). Figure 5.1 shows a gel image of ITS rRNA fragments amplified using the ITS rRNA PCR as published by White *et al* (Innis MA, Gelfand DH, 1990). All reactions had an input of 100ng template. Reduced amplification efficiency was observed for the *Aspergillus* species, with 25% (18/72) of the isolates failing to produce ITS rRNA products using the published primer sets. In figure 5.1 this is exemplified for several amplicons generated from *Aspergillus* isolates (figure 5.1), low intensity bands on gel electrophoresis indicate inefficient amplification in lanes 5, 7, 11 and 17.

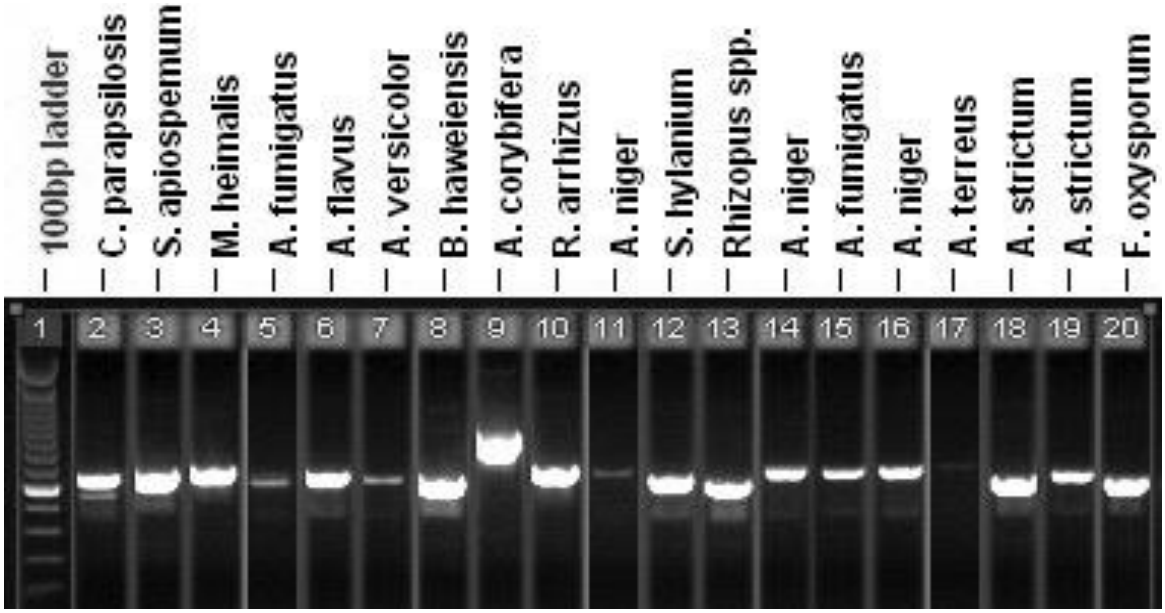



Figure 5.1 Example of an agarose gel for ITS rRNA amplicons obtained from fungal isolates using published primers by White *et al* (Innis & Gelfand, 1990).

### 5.3.3 Microscopic identification of fungal isolates

The overall identification rate compared with ITS rRNA sequenced based analysis was 80.3% (118/147) at the species level and 91.8% (135/147) at the genus level. Sub analysis demonstrated identification rates of 95.8% (71/73) at species level and 100% (73/73) at genus level for the *Aspergillus* species, table 5.1. For non-*Aspergillus* species the identification rates were 63%% (47/74) at species level and 84% (62/74) at the genus level, table 5.2.

Of the 19.7% (29/147) isolates not identified correctly to species level 5.4% (8/147) were identified at the genus level correctly but the investigator did not return the species level identification. For 6.1% (9/147) isolates the correct genus but incorrect species identification was reported, table 5.3. For 8.1% (12/147) of the isolates the genus was misidentified by the investigator, as detailed in table 5.3. The respective isolates misidentified at genus level included: *Myrothecium sp, Neofusicoccum sp, Pheosphaeriopsis sp, Pleosporales sp, Bionectria ochroleuca, Basidiomycete fungus (2), Alternaria alternata, Botryosphaeria stevensii, Mucor circinelloides, Mucor heimalis and Epicocum nigrum.*

**Table 5.1 Concordance between Microscopy and ITS rRNA identifications for *Aspergillus* isolates included in the study**

| | | Concordance (%) | | | |
|---|---|---|---|---|---|
| | | Microscopy | | MALDI TOF MS | |
| **Species** | ***n*** | **Species** | **Genus** | **Species** | **Genus** |
| *Aspergillus fumigatus* | 25 | 96 | 100 | 92 | 96 |
| *Aspergillus niger* | 19 | 100 | 100 | 100 | 100 |
| *Aspergillus oryzae/ flavus* | 9 | 100 | 100 | 100 | 100 |
| *Aspergillus terreus* | 5 | 80 | 100 | 80 | 100 |
| *Aspergillus versicolor /sydowii* | 5 | 100 | 100 | 80 | 100 |
| *Aspergillus nidulans* | 4 | 100 | 100 | 50 | 100 |
| *Aspergillus candidus* | 2 | 100 | 100 | 100 | 100 |
| *Aspergillus pseudoflectus/ustus/calidoustus* | 2 | 100 | 100 | 100 | 100 |
| *Aspergillus sclerotiorum* | 1 | 100 | 100 | 0 | 100 |
| **Total** | **73** | **95.9** | **100** | **90.4** | **98.6** |

**Table 5.2 Concordance between Microscopy and ITS rRNA identifications for non-*Aspergillus* isolates included in the study**

| | | Concordance (%) | | | | | | Concordance (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | | **Microscopy** | | **MALDI TOF MS** | | **Species** | | **Microscopy** | | **MALDI TOF MS** | |
| | ***n*** | **Species** | **Genus** | **Species** | **Genus** | | ***n*** | **Species** | **Genus** | **Species** | **Genus** |
| *Acremonium kiliense* | 1 | 0 | 100 | 0* | 0* | *Mucor racemosus* | 1 | 0 | 100 | 0* | 100 |
| *Acremonium strictum/alternatum* | 3 | 66.6 | 100 | 0 | 0 | *Mucor circinelloides* | 3 | 66.6 | 66.6 | 0 | 0 |
| *Alternaria alternate* | 2 | 50 | 50 | 100 | 100 | *Myrothecium sp* | 1 | 0 | 0 | 0* | 0* |
| *Unknown Basidomycete* | 2 | 0 | 0 | 100 | 100 | *Neofusicoccum sp* | 1 | 0 | 0 | 0* | 0* |
| *Bionectria ochroleuca* | 1 | 0 | 0 | 0* | 0* | *Paecilomyces lilacinus* | 1 | 100 | 100 | 100 | 100 |
| *Cladosporium cladosporioides* | 1 | 100 | 100 | 0 | 0 | *Paecilomyces variotti* | 1 | 100 | 100 | 0 | 0 |
| *Cochliobolus hawaiiensis* | 3 | 100 | 100 | 66.6 | 66.6 | *Penicillium chrysogenum* | 2 | 0 | 100 | 100 | 100 |
| *Botryosphaeria stevensii* | 1 | 0 | 0 | 0* | 0* | *Penicillium polonicium* | 1 | 0 | 100 | 0* | 100 |
| *Engyodontium album* | 3 | 100 | 100 | 0* | 0* | *Pleosporales sp* | 1 | 0 | 0 | 0* | 0* |
| *Epicoccum nigrum* | 1 | 0 | 0 | 0* | 0* | *Pleosporales sp* | 1 | 0 | 0 | 0* | 0* |
| *Exophiala spinifera* | 1 | 100 | 100 | 0* | 0* | *Rhizopus oryzae/arrhizus* | 3 | 100 | 100 | 100 | 100 |
| *Fusarium intermedius/proliferatum* | 2 | 0 | 100 | 50 | 50 | *Scedosporium apiospermum* | 6 | 100 | 100 | 100 | 100 |
| *Fusarium moniliforme* | 1 | 100 | 100 | 100 | 100 | *Scedosporium prolificans* | 1 | 100 | 100 | 100 | 100 |
| *Fusarium oxysporum* | 12 | 74.9 | 100 | 74.9 | 81.8 | *Scytalidium hylinum* | 1 | 100 | 100 | 0* | 0* |
| *Fusarium solani* | 6 | 83.3 | 100 | 100 | 100 | *Scytalidium dimidiatum* | 2 | 100 | 100 | 0* | 0* |
| *Fusarium sp* | 2 | 0 | 100 | 50* | 50 | *Scopulariopsis brevicaulis* | 1 | 100 | 100 | 100 | 100 |
| *Lichtheimia ramose* | 2 | 100 | 100 | 100 | 100 | | | | | | |
| *Mucor hiemalis* | 2 | 0 | 50 | 0* | 0 | | | | | | |
| *Mucor indicus* | 1 | 100 | 100 | 0* | 0 | *Total* | 74 | 63% (47/74) | 84% (62/74) | 54% (40/74) | 58% (43/74) |

| Table 5.3 Misidentification by microscopy compared with ITS rRNA sequenced based identification for 23 fungal isolates | | |
|---|---|---|
| **ITS rRNA identification** | **Microscopic identification** | ***n*** |
| **Species misidentification** | | |
| *Aspergillus terreus* | *Aspergillus alliaceus* | 1 |
| *Aspergillus fumigatus* | *Aspergillus candidus* | 1 |
| *Mucor racemosus* | *Mucor circinelloides* | 1 |
| *Fusarium proliferatum* | *Fusarium oxysporum* | 1 |
| *Fusarium solani* | *Fusarium oxysporum* | 3 |
| *Fusarium sp* | *Fusarium oxysporum* | 1 |
| *Acremonium sp/sclerotigenum* | *Acremonium kiliense* | 1 |
| *Total* | | 9 |
| **Genus misidentification** | | |
| *Myrothecium sp* | *Acremonium spinosum* | 1 |
| *Neofusicoccum sp* | *Scytilidium sp* | 1 |
| *Pheosphaeriopsis sp* | *Fusarium sp* | 1 |
| *Pleosporales sp* | *Scylalidium lignicola* | 1 |
| *Bionectria ochroleuca* | *Acremonium strictum* | 1 |
| *Basidiomycete fungus* | *Scytalidium hyalinum* | 2 |
| *Alternaria alternate* | *Ulocladium spp* | 1 |
| *Botryosphaeria stevensii* | *Scytilidium dimidiatum* | 1 |
| *Mucor circenelloides* | *Rhizopus stolonifer* | 1 |
| *Mucor heimalis* | *Rhizomucor variabilis* | 1 |
| *Epicocum nigrus* | *Aspergillus niger* | 1 |
| **Total** | | **12** |

### 5.3.4 Optimisation of SAB broth inoculation for MALDI-TOF MS

Prior to performing MALDI-TOF MS analysis on fungal cultures it was necessary to optimise the fungal concentration inoculated into Sabouraud's broth as over inoculation led to poor germination of the conidia. To optimize the inoculum three different cultures were prepared by taking a light, moderate and heavy sweep of the fungal colony surface. Figure 5.2 shows the results of this experiment after 24 hours incubation at 30°C on the rotating platform. The moderate inoculum produced optimal fungal biomass for analysis with well-developed fungal balls in suspension. The biomass in the light inoculum was not sufficient for protein extraction. The heavy sweep inoculum of conidia fungal balls was poorly developed and some un-germinated conidia were present that could interfere with the spectral consistency.

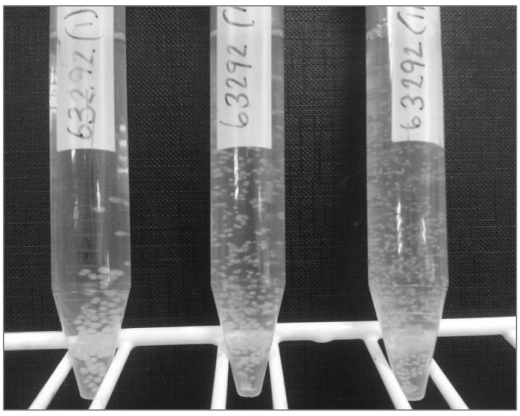


Figure 5.2 varying inocula (light, moderate, and heavy) for *Aspergillus fumigatus* fungal ball preparation for MALDI-TOF MS.

### 5.3.5 Optimisation of protein extraction for MALDI-TOF MS

Table 5.4 details MALDI-TOF MS results for four isolates processed using the three pellet drying methods detailed in section 5.2.10. Table 5.3 details the log similarity scores obtained for four isolates processed in triplicates through the three pellet drying conditions. In figure 5.3 log similarity scores are plotted for the three drying conditions.

**Table 5.3: MALDI TOF MS log similarity scores results obtained for four fungal isolates processed using three pellet drying conditions during protein extraction**

| | **Hot block** | | **Incubator** | | **No drying** | |
|---|---|---|---|---|---|---|
| ***n*** | **Identification** | **Score** | **Identification** | **Score** | **Identification** | **Score** |
| 1 | *A. oryzae/flavus* | 2.413 | *A. oryzae/flavus* | 2.283 | *A. oryzae/flavus* | 2.378 |
| | *A. oryzae/flavus* | 2.345 | *A. oryzae/flavus* | 2.347 | *A. oryzae/flavus* | 2.388 |
| | *A. oryzae/flavus* | 2.355 | *A. oryzae/flavus* | 2.279 | *A. oryzae/flavus* | 2.344 |
| 2 | *Aspergillus sp* | 1.926 | *A. fumigatus* | 2.32 | *A. fumigatus* | 2.487 |
| | *A. fumigatus* | 2.073 | *A. fumigatus* | 2.327 | *A. fumigatus* | 2.392 |
| | No peaks found | < 0 | *A. fumigatus* | 2.2 | *A. fumigatus* | 2.3 |
| 3 | *S. apiospermum* | 2.1 | no reliable ID | 1.6 | *S. apiospermum* | 2.1 |
| | *S. apiospermum* | 2.127 | *Scedosporium sp* | 1.935 | *Scedosporium sp* | 1.859 |
| | *Scedosporium sp* | 1.998 | *Scedosporium sp* | 1.982 | *S. apiospermum* | 2.217 |
| 4 | *F. oxysporum* | 2.311 | *F. oxysporum* | 2.185 | *F. oxysporum* | 2.218 |
| | *F. oxysporum* | 2.233 | *F. oxysporum* | 2.189 | *F. oxysporum* | 2.331 |
| | *F. oxysporum* | 2.277 | *F. oxysporum* | 2.318 | *F. oxysporum* | 2.295 |
| | **Median** | **2.18 (2.02, 2.34)** | **2.26 (2.03, 2.31)** | | **2.34 (2.21, 2.38)** | |

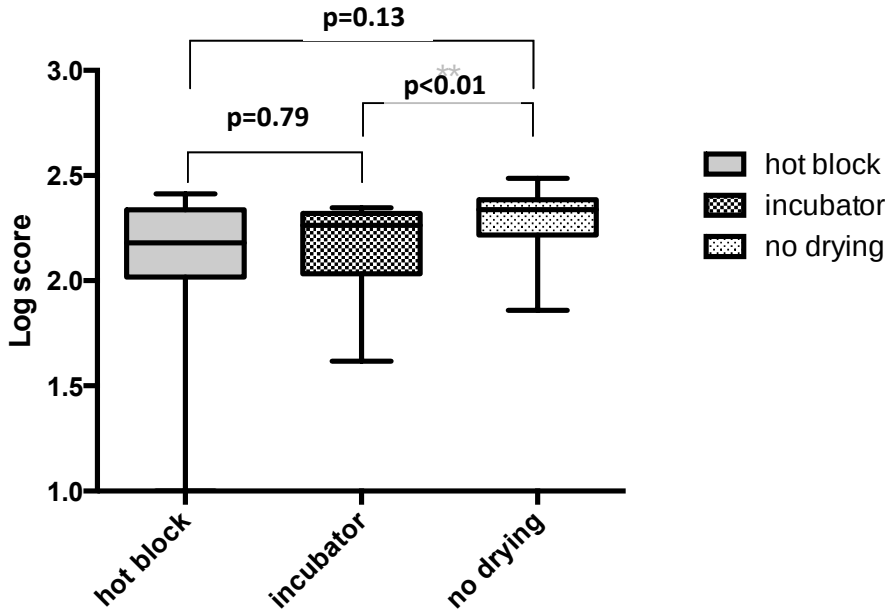



Figure 5.3 Box plot of log similarity scores obtained for four isolates (triplicate analyses) by MALDI-TOF MS using the pellet processing methods; a) drying in an incubator b) drying in a dry hot block c) no drying. Wilcoxon Signed Rank was used to calculate significance between the three methods.

No significant difference was observed between log similarity scores obtained for pellets that were not dried (condition *c*) compared with pellets dried in a hot block (condition *a*), with all isolates achieving species identification in condition *a* and *c*. A significant increase in log similarity scores was observed if pellets were not dried (condition *c*) compared to pellets being dried in an incubator (condition *b*), with one isolate not achieving species identification for condition b. As not drying the pellet (condition *c*) was shown to be most successful all subsequent analysis of fungi was performed without a drying step after ethanol dehydration.

### 5.3.1 MALDI TOF MS identification of fungal isolates

The overall identification rate by MALDI-TOF MS for the identification of fungal isolates was 71.4% (105/147) at species level and 78.9% (116/147) at the genus level. A result of 'no identification' with log similarity scores <1.7 was reported for 19% (28/147) of the isolates. 2.7% (4/147) isolates were misidentified at the species level with log similarity

scores >2.0 but all were identified to the correct genus level which was significantly lower than the misidentification rate of microscopy ($p<0.01$); One isolate was misidentified at the genus level with a log similarity score of 1.933 (table 5.4). Two isolates failed to produce MS spectra with a result of 'no peaks found' despite repeat analysis.

When limiting the analysis to species contained within the MALDI-TOF MS database the success rate was 83.3% (105/126) at species level, 92.1% (116/126) at genus level. Four of the isolates with a log similarity score >1.7 but <2.0 were correctly identified at the species level. 6.4% of the isolates with representative spectra in the database (10/127) had a result of 'no identification' with log similarity scores <1.7.

#### 5.3.1.1 Identification of *Aspergillus* species by MALDI-TOF MS

Sub analysis of MALDI-TOF MS identifications for the genus *Aspergillus* demonstrated 90% (67/73) success for identification at species level and 98.6% (72/73) at genus level, table 5.1. Four *Aspergillus* isolates with a genus only identification, log similarity score >1.7 but <2.0 returned the correct species identification. One *A. fumigatus* isolate identified as *A. sydowii* with a log similarity score of 1.793. This isolate phenotypically was identified as *A. candidus* by microscopy and was on further investigation shown to be a non-sporing variant of *A. fumigatus.* One *A. fumigatus* isolate by ITS rRNA analysis, obtained a result of 'no identification' log similarity score <1.7. Repeat analysis did not resolve this result despite representative spectra being present in the MALDI-TOF MS database.

#### 5.3.1.2 Identification of non-Aspergilus species by MALDI-TOF MS

The success rate for non-*Aspergillus* species was 54% (40/74) at the species level and 58% (43/74) to the genus level. A result of 'no identification' was returned for 37.8% (28/74) of the isolates, table 5.2. 6.76% (5/74) of the non-*Aspergillus* isolates were misidentified, and for 2.7% (2/74) of the isolates no peaks could be obtained during analysis despite repeat protein extractions being performed, including one *Curvularia hawaiiensis* and one *Engydontium* sp isolate.

#### 5.3.1.3 Isolates with a result of 'no identification' by MALDI TOF MS

Of the 28 non-*Aspergillus* isolates not identified by MALDI TOF MS 71.4% (20/28) did not have representative reference spectra present within the Biotyper database, indicated by the asterisk in table 5.2. The remaining 28.6% (8/28) were species for

which spectra were present in the reference database and remained unidentified including *Acremonium strictum* (2), *Cladosporium cladosporoides* (1), *Fusarium oxysporum* (2), *Mucor circineloides/rassimosis* (2) and *Paecilomyces varotii* (1).

#### 5.3.1.4 Misidentification of non-*Aspergillus* species by MALDI TOF MS

Table 5.4 details misidentifications of non-*Aspergillus* isolates by MALDI TOF MS. When applying manufacturers log similarity score thresholds, of >1.7 for genus identification and >2.0 for species identification, 6.8% (5/74) of non-*Aspergillus* isolates were misidentified in this study. Four isolates were correctly identified at genus level but misidentified at species level. One isolate was misidentified at genus level with a log similarity score >1.7, the respective isolate was identified as *Mucor sp* by ITS rRNA sequencing and *Rhizopus microsporus* by MALDI-TOF MS, both are mucoraceous fungi. If the acceptable species log similarity score was lowered to >1.7 three more isolates would have been misidentified, as detailed in section b) of table 5.4. All would have been correct at the genus level but incorrect at the species level.

**Table 5.4. Misidentifications by MALDI-TOF MS when a) applying log similarity score thresholds as recommended by the manufacturer b) accepting all identifications above a log similarity score of 1.7**

| | **ITS identification** | ***n*** | **MALDI TOF MS identification** | ***n*** | **LS score** |
|---|---|---|---|---|---|
| a) | *Curvularia hawaiiensis* | 2 | *Curvularia pallescens* | 2 | 2.147/2.198 |
| | *Penicillium polonicium* | 1 | *Penicillium chrysogenum/verrucosum* | 1 | 2.469 |
| | *Mucor hiemalis* | 1 | *Rhizopus microspores* | 1 | 1.933 |
| | *Mucor racemosus* | 1 | *Mucor circinelloides/ramosissimus* | 1 | 2.11 |
| b) | *Aspergillus fumigatus* | *1* | *Aspergillus sydowii* | 1 | 1.793 |
| | *Lichtheimia ramose* | *2* | *Lichtheimia corymbifera* | 2 | 1.83/1.854 |
| | **Total** | **8** | | **8** | |

LS score = log similarity score, ITS = intergenic spacer reigon

### 5.3.2 Applicability of log similarity score thresholds for MALDI TOF MS identification

The manufacturer's log similarity scores thresholds indicate identifications are acceptable at the genus level with log similarity scores 1.7-1.999 and to the species level with log similarity scores >2.0. Anything <1.7 is considered to be an unreliable

identification by the manufacturer. Table 5.5 details log similarity scores obtained across the genera included in this study alongside the frequency of reference spectra in the FFv1.0 database. Species that identify with high accuracy, such as the *Aspergillus* sp. are well represented in the reference database with the greatest proportion of reference spectra compared with non-*Aspergillus*, which are under represented, table 5.5. Four of the genera included in this study did not have representative reference spectra in the filamentous fungi library supplied by Bruker. The median log similarity score for correct identifications was 2.23 (IQR: 2.09-2.37). The median log similarity score for incorrect identifications was 1.31 (IQR 1.24-1.54). Figure 5.4 demonstrates the results obtained for the 147 isolates in this study according to their log similarity score and classified by colour according to the identification listed as the highest spectral match. Grey bars indicate the optimal spectral correlation that was correct at the species level and white bars indicate identifications that were correct at the genus level. Black bars indicate results where the best spectral match was a misidentification. 96.8% (92/95) of the isolates above 2.0 obtained the correct species identification. A further nine isolates would have been identified correctly if log similarity scores had been lowered to accept identifications >1.7 however, eleven isolates would have been misidentified at the species level.

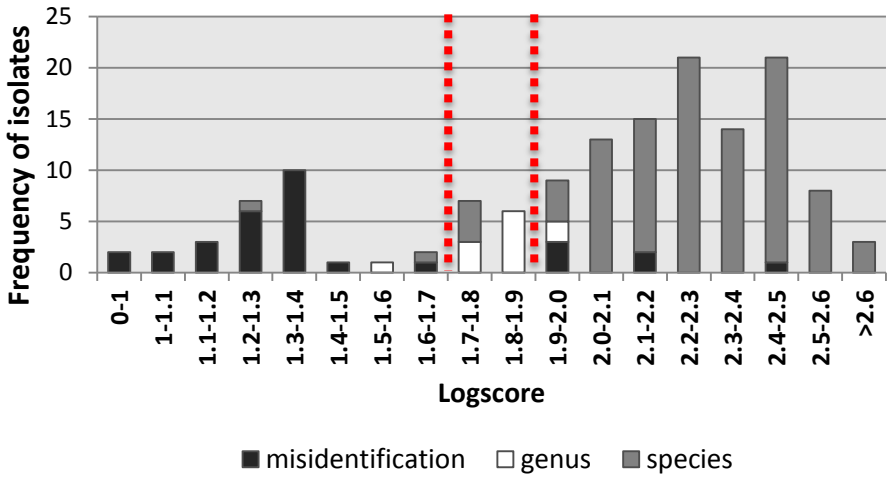



Figure 5.4 Histogram displaying the distribution of log similarity scores obtained for 147 isolates processed by MALDI-TOF MS. Grey bars indicate correct species, white indicates the correct genus and black indicates incorrect identification. Dashed lines indicate manufacturers log similarity score thresholds for genus and species identifications.

**Table 5.5. Log similarity score classification for 147 strains analysed by MALDI-TOF MS alongside the frequency of reference spectra for each genus contained within the FFv1.0 filamentous fungi Biotyper database**

| Genus | *n* | Species >2.0 | Genus 1.7-2.0 | No ID <1.7 | Mis-ID | No peaks | Reference Spectral (*n*) |
|---|---|---|---|---|---|---|---|
| Other | 5 | 1 | 1 | 1 | | | 165 |
| *Aspergillus* sp | 73 | 66 | 6 | 1 | | | 90 |
| *Penicillium* sp | 3 | 2 | | | 1 | | 67 |
| *Fusarium* sp | 23 | 16 | 2 | 5 | | | 36 |
| *Rhizopus* sp | 3 | 2 | 1 | | | | 15 |
| *Paecilomyces* sp | 2 | 1 | | 1 | | | 11 |
| *Lichthemia* sp | 2 | | 2 | | | | 11 |
| *Scopulariopsis* sp | 1 | 1 | | | | | 9 |
| *Scedosporium* sp | 7 | 7 | | | | | 6 |
| *Alternaria* sp | 2 | 2 | | | | | 5 |
| *Mucor* sp | 7 | | 1 | 5 | 2 | | 5 |
| *Cladosporium* sp | 1 | | | 1 | | | 1 |
| *Acremonium* sp | 4 | | | 4 | | | 1 |
| *Bionectria* sp | 1 | | | 1 | | | 1 |
| *Curvularia* sp | 3 | | | | 2 | 1 | 1 |
| *Cladosporium* sp | 1 | | | 1 | | | 1 |
| *Myrothecium* sp | 1 | | | | | 1 | 0 |
| *Neofusicoccum* sp | 1 | | | 1 | | | 0 |
| *Engydontium* sp | 3 | | | 3 | | | 0 |
| *Exophila* sp | 1 | | | 1 | | | 0 |
| Total | 147 | 98 (66.7) | 13 (8.8) | 29 (19.7) | 5 (3.4) | 2 (1.4) | 365 |

## 5.4 Discussion

This study has demonstrated that the MALDI-TOF MS and the FFv1.0 database can be utilised to identify fungi accurately with 71.4% (105/147) of isolates identified at species level and 78.9% (116/147) at the genus level. Identification was dependent on the database containing representative spectra for the species being identified. This identification rate is comparable with microscopy, which had an identification rate of 76.2% (112/147) at the species level in this study. However, significantly more isolates ($p<0.01$) 15.6% (23/147), were misidentified by microscopy compared with 3.4% (5/147) misidentified by MALDI-TOF MS. The FFv1.0 database was the first commercially available database for use in the diagnostic laboratory setting. Schulthess and colleagues (2014) recorded 78.3% and 54.2% success for the identification of fungi to the genus and species level respectively using the FFv1.0 database (Schulthess *et al.*, 2014). In a smaller study analysing non-*Aspergillus* isolates including; *Penicillium marneffei*, *Paecilomyces sp*, *Fusarium solani*, *Rhizopus sp* and *Pseudallescheria boydii* all isolates returned a result of 'no identification' with log similarity scores <1.7 despite some of the species being listed in the FFv1.0 database (Chen *et al.*, 2015). There are several factors that contributed to the lower success of fungal identification in the respective studies.

The success of MALDI-TOF MS for the identification of microorganisms is reliant on a robust reference database. In this study 19.7% (29/147) of isolates returned a result of 'no identification' despite repeat analysis. In the majority of cases the species being identified did not have representative spectra within the FFv1.0 database. Five isolates were not identified despite representative spectra being included in the database. This suggests that the reference spectra are not diverse enough to identify all strains of particular species. This has been observed in similar studies utilising databases known to contain representative spectra for isolates being analysed (Tam *et al.*, 2014; Chen *et al.*, 2015). The current FFv1.0 database is not comprehensive enough (in terms of breadth of reference spectra available) to suit the needs of a clinical mycology laboratory. By expanding the reference database the performance of MALDI-TOF MS will be significantly improved (Lau *et al.*, 2013; Becker *et al.*, 2014; Gautier *et al.*, 2014; Tam *et al.*, 2014). This is evident from studies choosing to utilise in-house collated databases for fungal identification. By far the most substantial in-house database to be created was the work of Gautier *et al* (2014). In a multi-centre approach genotypically characterized isolates were used to create a database consisting of 2832 spectra representing 347 species and 708 strains of fungi (Gautier *et al.*, 2014). The prospective identification of fungal isolates over a 16 month time frame demonstrated

98.8% (1094/1107) success for the identification of fungi in the routine diagnostic laboratory (Gautier *et al.*, 2014). In a second study utilising an in-house database containing 760 strains representing 472 species, prospective identification of clinical isolates demonstrated 95.4% success at the species level in the routine diagnostic setting (Becker *et al.*, 2014). Several other studies have also published relatively high success rates ranging between 87-100% using in house databases (Alanio, Beretti, *et al.*, 2011; Cassagne *et al.*, 2011; E De Carolis *et al.*, 2012; Ranque *et al.*, 2014; Sitterle *et al.*, 2014). Not surprisingly the performance of MALDI-TOF MS is improved for *Aspergillus* species, 81.5-100% (Sanguinetti and Posteraro, 2014), compared with non-*Aspergillus* species 0%-87.3% (Cassagne *et al.*, 2011; Lau *et al.*, 2013; Becker *et al.*, 2014; Ranque *et al.*, 2014; Chen *et al.*, 2015), confirming the observations in this study with 90% of *Aspergillus* isolates and 54% of non-Aspergilius isolates being identified. This is most likely a result of increased availability, interest in and access to *Aspergillus* strains during database construction which results in an over representation of reference spectra for *Aspergillus* isolates compared with non-*Aspergillus* species (Cassagne *et al.*, 2011; Lau *et al.*, 2013; Ranque *et al.*, 2014).

Building a comprehensive spectral reference database can also provide diagnostic laboratories with a method of identification with the accuracy and resolution similar that that of molecular identification methods. Studies have shown that MALDI-TOF MS was able to discriminate between the commonly isolated *A. flavus* and closely related rare cryptic species *A. nomius* and *A. tamari,* which in the laboratory cannot be distinguished phenotypically and would be misidentified. When *A. nomius* and *A. tamari* were analysed by MALDI-TOF MS these two cryptic species remained unidentified whilst *A. flavus* was identified. This demonstrates the spectral matching algorithm is powerful enough to distinguish between closely related cryptic fungal species and only provide an identification in the presence of representative spectra in the MALDI-TOF MS database for a given species (Tam *et al.*, 2014). This was further demonstrated by the discrimination between phenotypically identical species *Stachybotrys chartarum* and *S. chlorohalonata* (Gruenwald *et al.*, 2015) and *A. lentulus* from *A. fumigatus* (Verwer *et al.*, 2014) using MALDI-TOF MS. This means that if the MALDI-TOF database is comprehensively developed over time the laboratory will seldom have to rely on molecular methods or even the reference laboratory service to obtain an accurate identification of moulds from invasive infections. Ultimately both the epidemiology of fungal infection and the understanding of the pathogenesis of fungal disease and treatment regimens will be enhanced through accurate identification. Collaboration between networks of centres in establishing a comprehensive national

collection of spectra may be one way of rapidly improving and standardising local mycology services.

There are several other parameters that contribute to the improved success of identification using MALDI-TOF MS databases for fungal identification. Accurate identifications are obtained by MALDI-TOF MS when the processing method used to create the reference database entries is also used to process unknown cultures (Lau *et al.*, 2013; Becker *et al.*, 2014; Chalupová *et al.*, 2014). Consistency between the reference spectra and unknown isolates provides the best conditions for a match. The FFv1.0 database is composed of reference spectra created using the liquid cultivation method (Maier and Kostrzewa, 2009). In this study the liquid cultivation method was adhered to with the exception of one variation, the removal of pellet drying after ethanol dehydration. The standard operating procedure published by the manufacturer recommends that the pellet should be dried completely in a vacuum concentrator or at 37°C for 5-10 minutes prior to formic acid extraction, and emphasizes the importance of completely drying the pellet. In our centre we do not have access to a vacuum concentrator. In our experience when pellets were incubated for 5-10 minutes at 37°C very little moisture evaporated and pellets were not dry. Two alternative approaches were investigated, extending the incubation at 37°C to two hours or using a dry hot block to evaporate the remaining moisture from pellets. The performance of MALDI-TOF MS was not enhanced by either of these approaches. When drying was omitted from processing a significant increase in log similarity scores was observed and 93.8% (15/16) of the technical replicates were identified with log similarity scores >2.0.

In one study utilising vacuum centrifugation after ethanol dehydration, lower success rates of 78.3% at the genus level and 54.2% at species level were observed despite the majority of species in this study being represented in the FFv1.0 database. Schulthess *et al* observed 13.3% (38/285) of their analyses obtaining a log similarity score between 1.7-2.0 (Schulthess *et al.*, 2014) compared with 8.8% (13/147) in this study. Log similarity scores are in part determined by peak frequency and intensity within spectra. Higher intensity and increased frequency of matched peaks contributes to a higher log similarity score. If protein extraction is optimal it would be expected that isolates with representative spectra in the reference database would achieve an identification with a log similarity score >2.0. Poor spectral quality through sub optimal protein extraction could explain why isolates are not identified despite the species being covered by the highest number of reference entries in the reference database (Becker *et al.*, 2014; Schulthess *et al.*, 2014). It is possible that the drying of pellets so intensely is having a negative impact on analysis. In written communication with the

manufacturer it was disclosed that vacuum centrifugation is being performed so vigorously in some laboratories that fungal biomass is being transformed into hardy dry pellets that are difficult to re-suspend when formic acid is added. The rationale behind drying the pellets is to reduce the size so that a reasonable amount of formic acid can be added, i.e. 50µL. In our study this was resolved by adding 100µL of formic acid and the same volume acetonitrile for large fungal pellets which on analysis led to log similarity scores >2.0. Cassagne *et al* do not report using a drying step during the preparation of protein extracts from colony scrapes of fungal biomass with high success and log similarity scores >2.0 across the majority of analyses (Cassagne *et al.*, 2011). Additionally a further recommendation made by the manufacturer is that fungal identifications are only accepted if the log similarity score falls within thresholds set by the manufacturer (as detailed in section 2.3). Thresholds are applied to the log similarity score to ensure a match is sufficiently powerful enough to be accepted at the genus or species level. Often log similarity scores for fungal analysis fall below those thresholds despite the correct fungus being indicated as the best match in the reference database.

An alternative approach for validating identifications is to disregard the use of log similarity scores and instead measure concordance of spectral matching across replicates of the same protein extract. By analysing protein extracts in quadruplicate identifications can be accepted if three out of four results give the same spectral match with the reference database. Cassagne *et al* pioneered this approach reporting 98.7% success for isolates contained within their in-house reference database (Cassagne *et al.*, 2011). Becker and colleagues adopted this rationale in a subsequent study and reported 95.4% success to the species level with log similarity scores ranging from 1.22-2.62. Notably in the respective study only 85.6% success would have been achieved utilising a log similarity score threshold of >1.7 for species identification (Becker *et al.*, 2014). In 2014 Gautier *et al* adapted this identification rule slightly to accept an identification if three of four analyses had the same spectral match and at least one of the matches had a log similarity score of >1.9 (Gautier *et al.*, 2014). The respective study reported the most successful identification rate with 98.8% of isolates being identified to species level. In our study protein extracts were analysed in duplicates and only one result with a log similarity score >2.0 was required for the identification to be accepted. Decreasing the log similarity score to >1.9 for species identification would have led to the misidentification of twelve isolates including two *Lichthemia ramosa* isolates as *Lichthemia corymbifera*. The function of the log similarity score threshold for species identification (>2.0) is to prevent misidentification

when the match between reference spectra and an unknown isolate is not optimal. The anti-log of genus thresholds (log similarity score 1.7-2.0) only equates to an actual score between 50-100 out of a possible 1000, derived in part from matched peaks and their relative intensity to unmatched peaks within the spectra. Lowering the log similarity score for species identification allows fewer or less intense peaks to be required for a match and thus weakens the power of identification. By maintaining the log similarity score thresholds set by the manufacturer stringency is maintained and limits the number of misidentifications, as demonstrated in this study. The ultimate goal should not be to discontinue use of log similarity scores but to optimize protein extraction to obtain high log similarity scores for those species represented in the database. Across all published MALDI-TOF MS fungal studies the protein extraction methods are less than standardised and may be contributing to lower log similarity scores obtained using the Biotyper software.

When a fungal processing method is used other than the method used to create the reference database it leads to poor performance of MALDI-TOF MS. This was demonstrated in two studies utilising the FFv1.0 database. Lau *et al* processed fungal colonies by excising biomass from agar and placing in 100% ethanol with zirconia silica beads and vortexing for 15 minutes (Lau *et al.*, 2013). The suspension was centrifuged and pellet suspended in 70% formic acid and again vortexed for 5 minutes. Acetonitrile was added and the supernatant was utilised for analysis. Analysis of 421 clinical isolates demonstrated 0.7% success at the species level (log similarity score >2.0) using the FFv1.0 database. When spectra were analysed against the Lau in-house database created using the same processing method to create reference spectra and to prepare isolates 88.9% success was achieved at species level (log similarity scores >2.0) (Lau *et al.*, 2013). Chen *et al* utilised the colony scrape method for transferring biomass into 75% ethanol. Conidial suspensions were centrifuged then washed with ethanol then dried for 1 hour prior to formic acid extraction (Chen *et al.*, 2015). Using the respective method all analyses returned log similarity scores <1.7 when analysed against the FFv1.0 database however, the correct identification was listed as the best match by the Biotyper software for all isolates indicating that the spectra from the conidial suspensions were of limited similarity to reference spectra in the Biotyper database but the software was still able to match the isolates with the correct species identification. The creation of an in-house database using the colony scrape method also led to the successful identification of 28 *Penicillium marneffii* isolates with log similarity scores ranging from 1.83-2.45 (Chen *et al.*, 2015).

The colony scrape method, especially when being used for hazard group 3 fungi, has some limitations. Scraping colonies with a loop or other inoculating device will liberate conidia into the environment. This task must be conducted within an isolated environment within a biological safety cabinet. Conidia can also be hydrophobic making them difficult to centrifuge (Wosten and de Vocht, 2000; Grunbacher *et al.*, 2014) and as with any other microbial suspension the vortexing of conidial suspensions presents a health and safety risk, especially in the instance of hazard group 3 fungi. When creating mass spectrum profiles from fungal colonies variation in the protein profiles is apparent (Chalupová *et al.*, 2014). Young and mature colonies produce different spectra (Alanio, Beretti, *et al.*, 2011), presumably due to the production of fruiting structures and conidia in mature colonies, with the most reproducible spectra being obtained from cultures grown for 120h compared with those at 48, 72 and 96 hours (Del Chierico *et al.*, 2012). If the age of the colony being analysed is not similar to the age of the colony used to create the reference spectra it will contribute to a poorer match. The respective issues are partially resolved with the use of liquid cultivation.

In this study we have demonstrated that only a light inoculum of conidia/fungal biomass needs to be harvested onto a wet cotton swab, which was conducted inside a plastic bag to reduce the spread of conidia. Manipulating cultures inside a biological safety cabinet is desirable but not essential as the sample bag can be used as an alternative. The swab was then directly inoculated into SAB broth, expressed and the culture sealed. After 24 hours of incubation the resulting biomass manifests as mycelial balls, as the production of fruiting structures and conidia are suppressed through the rotation of the cultures preventing an aerobic interface from forming. Although this extends the turnaround time by 24 hours, mycelial balls are of much lower risk throughout processing than conidial suspensions. One observation made throughout the study was that not all fungal species produce classic fungal balls. Mucoraceous fungi produce large mycelial masses, much larger than what is required for analysis, thus need some degree of division prior to analysis. There may also be an opportunity to shorten the incubation time for mucoraceous fungi to achieve and identification earlier. Wet fungi such as *Fusarium* sp produce a hazy suspension that at first analysis appears to be contamination of the SAB broth, however on analysis this is germinated conidia of wet fungi. One potential drawback is that SAB liquid cultures may become contaminated at the point of inoculation that is only realised upon obtaining the MALDI-TOF MS identification, typically *Aspergillus fumigatus* contamination of a non-*Aspergillus* preparation. In our centre we observed very little contamination by

manipulating the fungal cultures inside a sample bag, using careful technique and ensuring fungal cultures were pure.

It has also been demonstrated that fungal pigments, including endogenous melanin, inhibit MALDI TOF MS analysis (Buskirk *et al.*, 2011). An absence of peaks was observed through spectra obtained from *Aspergillus niger* preparations extracted from a typical mature sporing colony. If pigmented fungi are sub-cultured onto specialist media such as malt extract agar in the instance of Stachybotrys (Gruenwald *et al.*, 2015) or a media containing tricylazole (Buskirk *et al.*, 2011) non-melanised colonies can be cultured and analyzed by MALDI TOF MS. However, these sub cultures require several days to develop. The alternative is liquid cultivation. If *A. niger* is grown in liquid culture abundant peaks are obtained from protein extracts, as preparations do not contain the highly melanised conidia (Buskirk *et al.*, 2011). The analysis of dematiaceous fungi was not inhibited with almost all protein extracts from pigmented fungi achieving quality spectra were obtained with the exception of one *Curvularia hawiiensis* isolate. Failure to identify dematiaceous fungi using the colony scrape method is documented in several studies presumably due to the presence of highly melanised fungal elements (Cassagne *et al.*, 2011; Becker *et al.*, 2014). In this study we followed the prescribed processing and analysis methods as recommended by the manufacturer so that the performance of the FFv1.0 database for use in the routine diagnostic setting could be fully evaluated. Despite adhering to all recommended parameters several isolates were misidentified.

Four isolates were misidentified all achieving log similarity scores >2.0. For the respective isolates the correct genus was indicated but the species identification was incorrect. This has been previously described using MALDI-TOF MS when identifying closely related bacterial species such as *Streptococcus pneumoniae* and the *Streptococcus mitis* group or *Escherichia coli* and *Shigella sp* (Werno *et al.*, 2012; Schaumann *et al.*, 2013). These species cannot be discriminated at the species level using the current Biotyper database (v3.1). The same may be true for some fungal species. The misidentification at the species level for fungi has been described across studies utilising MALDI-TOF MS (Cassagne *et al.*, 2011; Becker *et al.*, 2014; Schulthess *et al.*, 2014). The impact of misidentification cannot be fully estimated until the use of this technology advances in mycology laboratories and a comprehensive reference database is developed for fungal identification. Despite limited misidentifications there was a significant improvement in accuracy of the identification in this study using MALDI-TOF MS compared with conventional microscopic

techniques, which was also demonstrated in several other studies (Becker *et al.*, 2014; Gautier *et al.*, 2014). Fungal identification in our routine diagnostic setting is not satisfactory with 15.6% (23/147) of isolates misidentified by microscopy. 60.7% (14/23) of the misidentifications were isolates identified correctly at the genus level but an incorrect species reported. For the remaining 39.3% (9/23) rarely encountered fungi were misidentified as common pathogenic species.

The misidentification of fungi by conventional microscopy has been reported in several other studies to be between 19.9-38.5% (Ferreira *et al.*, 2013; Becker *et al.*, 2014; Gautier *et al.*, 2014; Ranque *et al.*, 2014) overall when compared against gold standard molecular identification. With the advent of molecular diagnostic assays species, such as *A. fumigatus,* are being classified into multiple distinct genotypically different species that also demonstrate varying susceptibility patterns to front line antifungal agents (Verwer *et al.*, 2014). Therefore the accurate identification of the new species is important both pathogenically and pharmacologically to the patient.

When reanalysing isolates identified with conventional microscopy up to 24% of *A. fumigatus* isolates were reclassified as *A. lentulus* and up 51% of *A. niger* isolates were shown to have been misidentified (Balajee *et al.*, 2006; Hendrickx, Beguin and Detandt, 2012). In this study rare fungi including *Myrothecium sp* were misidentified as commonly encountered fungi that are microscopically similar. One striking observation was the misidentification of two *Thanatephorus cucumeris* isolates as *Scytalidium hyalinum.* Microscopy images are demonstrated in figure 5.5 for a) *S. hyalinum* and b) *Thanatephorus cucumeris.*

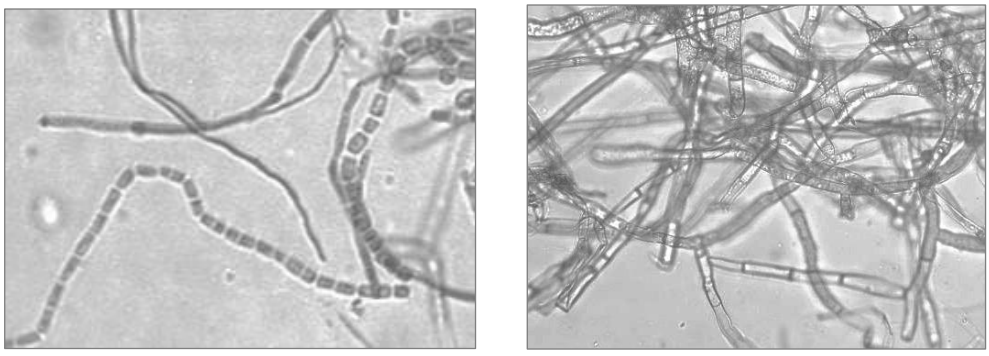


Figure 5.5 Microscopy features for a) *Scytalidium hyalinum* and
b) *Thanatephorus cucumeris* demonstrating hyaline hyphae with arthroconidia.

The two *Thanatephorus cucumeris* isolates when cultured under laboratory conditions rapidly grew as spreading white colonies with aerial mycelium and arthroconidia seen

on microscopy, similar to colonies of *S. hyalinum*. In our laboratory we do not frequently encounter *S. hyalinum*. Upon suspicion of isolating this species we now rely on molecular methods for accurate identification, as the clinical consequences of a misdiagnosis of onychomycosis from this fungus are considerable, including possible surgical debridement of infected tissue. In this study MALDI TOF MS was able to identify these two isolates as *Thanatephorus cucumeris* and would have been a faster and cheaper alternative to molecular methods.

The implementation of MALDI-TOF MS for fungal identification is now reliant on the expansion of commercially accessible databases by the manufacturer or by referral to experienced clinical mycology centres. It is essential that reference spectra meet quality criteria for inclusion in the database being utilised for routine use. Until this happens implementation of the current FFv1.0 database is not possible due to the high rate of isolates that are unidentified (Schulthess *et al.*, 2014). A result of 'no identification' could be used as a mechanism to prompt the use of molecular techniques for identification but this approach warrants further prospective investigation. Reference spectra would then be created for the isolates for inclusion in either a local or national database collection. In summary MALDI-TOF MS has clearly demonstrated the potential to revolutionize clinical mycology services in the near future as efforts are directed towards utilising this technology for fungal identification.

# Chapter 6 Improving the diagnosis of Candidemia: evaluation of Gram's stain and PNA FISH for the rapid identification of yeasts from blood culture

## 6.1 Introduction

The most important diagnostic test applied to a positive blood culture is a Gram's stain from which yeasts can be detected (Thairu, Usman and Nasir, 2014). Yeasts stain Gram positive owing to the selective capture of the crystal violet between the layers of the cell wall as it complexes with the iodine component of the staining process (Henrici, 1914). Gram's stain is rapid with results available to clinicians in less than 15 minutes from the point at which a blood culture is known to be positive. This provides clinicians with essential information allowing targeted management of patients with antifungal agents but is limited to generalising the identification to that of 'yeasts seen'. In the last century the utility of Gram's stain for yeast identification has not advanced. The size and shape of yeasts are easily distinguished from those of bacterial cells, as they are almost ten times larger with distinctive morphological characteristics such as blastospores, hyphae and pseudohyphae.

These morphological characteristics of yeasts are distinctive and may offer an opportunity to further identify yeast cells within a Gram's stain preparation. Harrington *et al* detailed the use of yeast morphology in Gram's stain to identify *C. albicans* from non-albicans species (Harrington *et al.*, 2007). The investigators utilised aggregates of pseudohyphae, a common feature within blood cultures from patients with *Candida albicans* fungemia, to achieve *C. albicans*/non-albicans identification. Authors demonstrated a sensitivity, specificity, positive predictive value and negative predictive value of 85%, 97%, 96%, and 89% respectively for *C. albicans* identification from Gram's stain (Harrington *et al.*, 2007). This study is the only published work investigating this method of speciation from Gram's stain for yeasts. It may be possible to further utilise morphological feature of yeasts within Gram's stain preparations for species identification and this technique may not just be limited to the gross differentiation of *C. albicans* from other species. However, in recent years an alternative approach to Grams stain has been advancing which may offer more specificity through genotypic identification of yeasts direct within microscopy sample preparations.

Fluorescence *in situ h*ybridisation (FISH) utilises target -specific probes coupled with wavelength-specific fluorogenic molecules allowing for visualisations using a fluorescence microscope. In-house developed FISH methods can be complex and require specialist training and expertise to perform (Bisha, Kim and Brehm-Stecher, 2011). For diagnostic laboratories standardization of the process is desirable. The release of a commercially available *C. albicans* PNA FISH assay 12 years ago allowed for the translation of FISH into the diagnostic setting (AdvanDX). In 2005 Wilson and colleagues published the first evaluation of the assay in a multi-centre study analysing 244 yeast blood cultures (Wilson *et al.*, 2005). The overall performance of the assay was 99%, 100% 100% and 99.3% for sensitivity, specificity, positive and negative predictive values respectively. Only one *C. albicans* isolate was undetected on first attempt out of 97 isolates and on repeat the blood culture was correctly reported positive (Wilson *et al.*, 2005). The PNA FISH single target assay was also demonstrated to be beneficial economically by enabling targeted antifungal therapy. Alexander *et al* (2006) *et al* demonstrated a cost saving of $1837 per patient after implementation for the *C. albicans* probe in their patient care pathway. Savings were realised by switching to fluconazole instead of caspofungin (Alexander *et al.*, 2006).

In the second version of the assay PNA FISH was further developed to include multiplex probes combined with different fluorophores, a green fluorescing PNA probe complementary to *C. albicans* and red fluorescing PNA probe complementary to *C. glabrata*. The probe multiplex demonstrated 98.7% (78/79) and 100% (37/37) sensitivities for the identification of *C. albicans* and *C. glabrata* respectively (Shepard *et al.*, 2008). Specificity for both probes was 100% (82/82). The PNA probe multiplex was tested against 80 reference strains encompassing 33 yeast species (Shepard *et al.*, 2008). Cross-reaction of the PNA probes was only reported with closely related species within the *C. glabrata* clade (*C. nivariensis* and *C. bracariensis*). Only one false negative result was reported for a *C. albicans* blood culture, which on repeat was positive (Shepard *et al.*, 2008). In a third version of the YTL PNA FISH assay the multiplexing capabilities of the assay were further expanded to include five PNA probes labelled with three fluorophores targeting *C. albicans/C. tropicalis* (green), *C. parapsilosis* (yellow) and *C. glabrata/krusei* (red). At the time of study the third version of the assay had not been evaluated for its diagnostic utility in the laboratory. In this study Gram's stain analysis and PNA FISH YTL V.3.0 will be evaluated for the identification of yeasts direct from blood culture.

## 6.2 Methods

### 6.2.1 Study design

My role in this study was as the principal investigator, having the technical responsibility for artificial inoculation of blood cultures and distribution of positive samples to three investigators who were blind to the yeast identification within the blood culture samples.

- Investigator 1 was trained to an advanced level (four months training) for the identification of yeasts from Gram's stain through analysis of yeast morphology in blood culture smears. The respective investigator performed Gram's stain analysis with the aid of identification keys (see figure 6.1 for an example identification key).
- Investigator 2, with no experience in Gram's stain analysis for yeast identification, analysed the slides using the identification keys.
- Investigator 3, who had received three days training prior to the study, performed PNA FISH Yeast Traffic Light (YTL) analysis.

Results were submitted to the principal investigator for analysis. When discrepancies with PNA FISH YTL were observed the investigator repeated the PNA FISH YTL analysis on the original preparation.

### 6.2.2 Yeast isolates

Yeast isolates from previous cases of fungaemia were selected to represent a diverse collection of clinical isolates. Isolates were stored at the Royal Free Hospital NHS microbiology laboratory. Isolates had previously been identified by Auxacolor2 biochemical analysis by the diagnostic laboratory.

### 6.2.3 Simulated blood cultures:

See general methods 2.1.3. Blood cultures were prepared as mono-fungal cultures (n=50) with the exception of two mixed cultures prepared as an additional analysis to assess the performance of each method for mixed cultures.

### 6.2.4 ITS rRNA sequenced based identification of yeasts

See general methods 2.2.

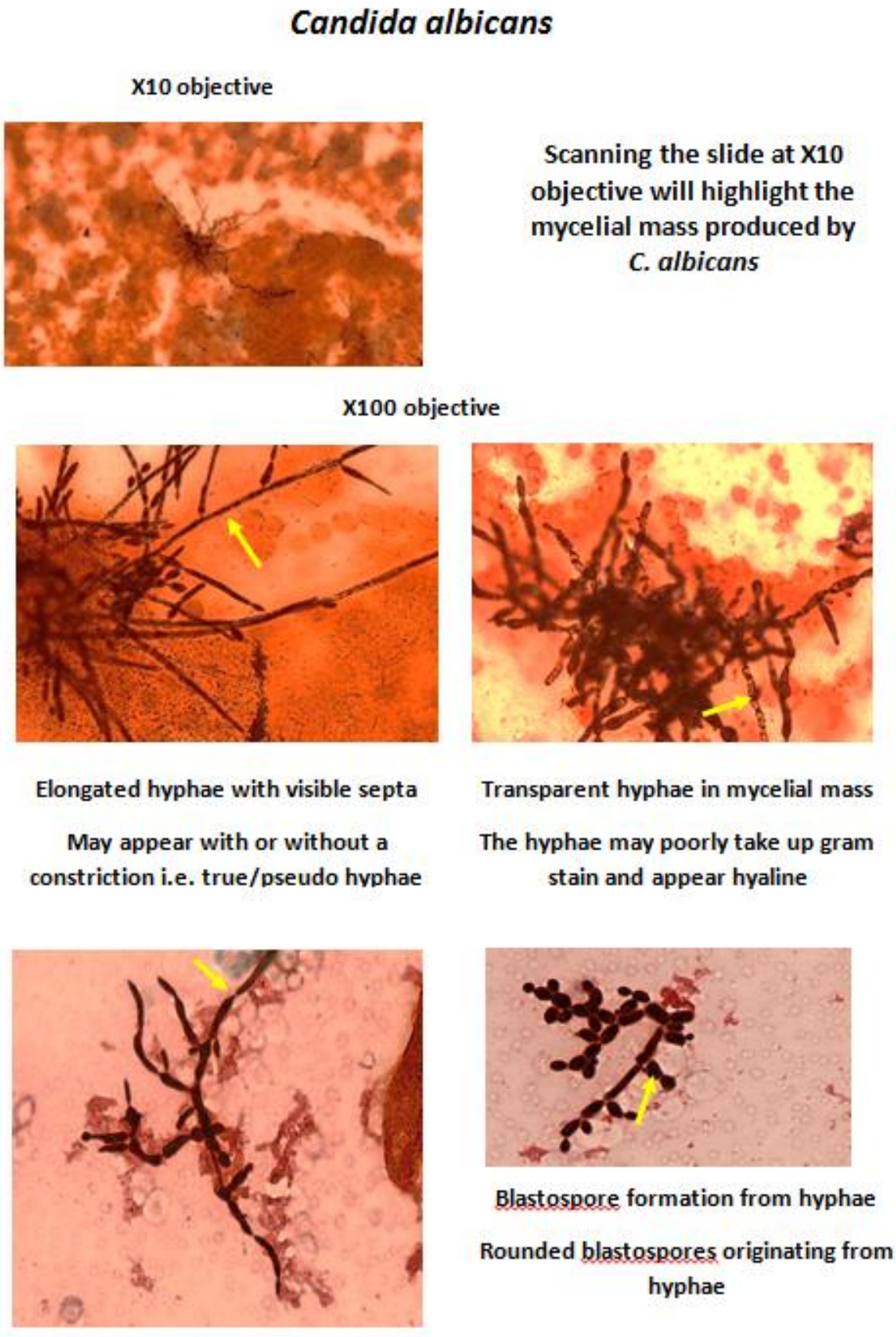



Figure 6.1 Example of an identification key used for Gram's stain analysis of *Candida* albicans from blood cultures

### 6.2.5 Gram's stain

Blood smears were fixed with absolute ethanol and Gram's stain (Sigma Aldrich, USA) method was applied (26). The principal investigator created identification keys for the seven most commonly isolated yeast species including: *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. neoformans, C. guilliermondii and C. parapsilosis*, see figure 6.1 for the *C. albicans* example. Identification keys were generated from analysis of spiked blood cultures. For each of the seven species included in the identification keys an American Type Culture Collection (ATCC) control strain and five clinical isolates were used to create simulated blood cultures, with the exception of *C. dubliniensis,* as no ATCC control strain could be acquired in suitable timescale. For this species a *C. dubliniensis* strain characterized by the Public Health England reference laboratory was utilised. Two sets of Gram's stain slides were prepared from each positive blood culture, totalling 12 slides from 6 blood cultures per species. The resulting 12 slides (per species) were analysed and consistent morphological characteristics were determined for each species. Images were captured at varying magnifications. Each identification key included colour images plus descriptive terminology to aid identification. Identification characteristics included the presence or absence of pseudo hyphae, clustering or branching of pseudo hyphae and the size and shape of the blastospores compared against the size of the red blood cells within the smear. If a yeast could not be identified the reviewer was instructed to report this as 'unknown'. Each analysis was timed by the reviewer from the start of preparing the Gram's stain to reporting the result.

### 6.2.6 PNA FISH

One drop of blood was added to each PNA FISH slide and heat fixed with fixation solution for 20 minutes on a slide station at 55°C. A drop of PNA Traffic Light probe was added to each slide and hybridised at 55°C for 30 minutes. The slides were then washed for 30 minutes in a buffered wash solution in a water bath set to 55°C to remove excess unbound probe. Slides were air-dried and mounting medium was added and then a coverslip applied. For each round of analysis a control slide, supplied by the manufacturer, was prepared. The slides were analysed using fluorescent microscopy. Visualisation of green fluorescence indicated the presence of *C. albicans* or *C*. *parapsilosis*, red fluorescence indicated *C. glabrata* or C. *krusei* and yellow fluorescence indicated *C. tropicalis*.

### 6.2.7 Antifungal impact assessment

The clinical registrar collaborating in this study carried out a retrospective review of clinical notes of patients from whom the yeasts included in this study had been isolated. Predicted choice of antifungal therapy was estimated on the basis of the yeasts susceptibility to fluconazole in that identifying *C. albicans*, *C. parapsilosis*, or *C. tropicalis* would have resulted in commencing fluconazole therapy, and identifying *C. glabrata* or *C. krusei* would have prompted the use of Caspofungin as initial therapy (Stone *et al.*, 2013). The actual antifungal therapies the patient received versus the antifungal that may have been selected based on the PNA FISH results were compared and an impact on antifungal choice estimated from the comparison.

### 6.2.8 Evaluation of assay parameters

The cost per test was determined based on reagent cost alone per sample (labour and indirect costs not included in analysis). Turnaround time (TAT) was measured across three repetitions of processing, one sample for each method and the mean TAT calculated. Technical ability (as defined by the level of training required to perform the assay) was assessed for each method based on the qualitative data produced. For PNA FISH analysis a scientist with 3 days training provided by the principal investigator undertook the testing. This scientist had no previous experience with the PNA FISH assay. Training was considered to be minimal. For the Gram's stain the technical ability was determined from comparing the performance of the 'advanced' scientist using the key cards to that of the 'inexperienced scientist'.

## 6.3 Results

### 6.3.1 Yeast identification from CHROMagar® *Candida* cultures;

Table 6.1 details yeast identifications obtained by ITS rRNA analysis from CHROMagar cultures for yeasts selected for inclusion in the study including; *C. albican*s (17/50), *C. glabrata* (9/50), *C. tropicalis* (4/50), *C. krusei* (4/50), *C. parapsilosis* (7/50), *C. orthopsilosis* (1/50), *C. metapsilosis* (1/50), *C. guilliermondii* (2/50), *Cryptococcus neoformans* (2/50), *Magnusiomyces capitatus* (1/50), *Cyberlindnera fabianii* (1/50).

| Table 6.1. ITS rRNA identifications for yeasts spiked into blood cultures | | | | | |
|---|---|---|---|---|---|
| **Species** | **%** | ***n*** | **Species** | **%** | ***n*** |
| *C. albicans* | 34 | 17 | *Cryptococcus neoformans* | 4 | 2 |
| *C. glabrata* | 18 | 9 | *Magnusiomyces capitatus* | 2 | 1 |
| *C. parapsilosis* | 14 | 7 | *Cyberlindnera fabianii* | 2 | 1 |
| *C. krusei* | 8 | 4 | *C. metapsilosis* | 2 | 1 |
| *C. tropicalis* | 8 | 4 | *C. orthopsilosis* | 2 | 1 |
| *C. guilliermondii* | 4 | 2 | | | |

Table 6.2 displays the results for Gram's stain and PNA FISH analysis for the 50 monofungal blood cultures. Analysis of performance is displayed in three categories, a) overall success b) success of identifying those species only contained within the assay database and c) success of identifying those species only contained within the assay database including unknown as the correct result.

**Table 6.2 Performance of Gram's stain and PNA FISH for the identification of yeasts direct from blood cultures with sub analyses based on species contained within the assay formats/databases**

| | | **Subset description** | | | | **Classification results** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Species *n*** | **Total *n*** | **%** | | **Correct** | **MisID[$]** | **Unknown** | **Success %** |
| (a) | Performance total | | | | | | | | |
| | Gram's stain | 10 | 50 | 100 | | 36 | 13 | 1 | 72 |
| | PNA FISH | 10 | 50 | 100 | | 43 | 2 | 5 | 86 |
| (b) | Performance only on species in respective method database | | | | | | | | |
| | Gram's stain | 7 | 47 | 94 | | 36 | 10 | 1 | 76.6 |
| | PNA FISH | 5 | 43 | 86 | | 43 | 0 | 0 | 100 |
| (c) | Performance on species in respective method database (including 'unknown' as the correct identification) | | | | | | | | |
| | Gram's stain | 10 | 50 | 100 | | 36 | 13 | 1 | 72 |
| | PNA FISH | 10 | 50 | 100 | | 48 | 2 | 0 | 96 |

MisID = misidentification

### 6.3.2 Gram's stain

Overall correct identifications, when compared with ITS rRNA sequencing, for Gram's stain analysis by the advanced investigator was 72% (36/50), which remained relatively unchanged across the three analyses, table 4.1. A slight increase in the success rate was observed when parameters were limited to more common species that were contained in both databases. Table 4.2 details the misidentifications made by the advanced investigator in 26% (13/50) of the cultures. Of the misidentifications 75% (3/4) of the *C. krusei* isolates were misclassified as *C. parapsilosis*. *C. albicans* was misidentified twice as *C. tropicalis* and once as *C. parapsilosis.* 'Missed' *y*east was reported for one blood culture. Mixed culture analysis led to further misidentification with both mixed cultures being reported as C. parapsilosis. The correct species was not reported from either bottle and the mixed culture was undetected. Gram's stain analysis performed by the second inexperienced investigator returned a success rate of 40% (20/50).

### 6.3.3 PNA FISH

Of the monofungal yeasts blood cultures 86% (43/50) of the yeast isolates were identified by the PNA FISH YTL assay. 100% (43/43) success was observed for the identification of yeast species contained within the assay's database and 96% (48/50) success when including 'unknown' as the correct result for yeasts not included in the PNA FISH YTL assay format table 4.1.

From the analysis of mixed cultures one *C. glabrata* isolate was not detected in a mixed culture with the *C. tropicalis* isolate being missed. The second mixed culture was correctly identified to contain *C. albicans* and *C. tropicalis*.

Misidentifications by PNA FISH are displayed in table 6.3. Two false positives for the *C. glabrata/C. krusei* (red) probe were observed one with *Cryptococcus neoformans isolate* and the other with a *Blastoschizomyces capitatus* isolate. On repeat both cultures were recorded as negative. The *C. metapsilosis* and *C. orthopsilosis* isolates were identified as *C. albicans*/*C. parapsilosis* with green fluorescence seen on microscopy.

**Table 6.3 Isolates misidentified or not identified from blood cultures by Gram's stain, PNA FISH and MALDI-TOF compared with the expected result.**

| | | Misidentifications / blood cultures not identified | | | |
|---|---|---|---|---|---|
| **Expected result** | ***n*** | **Gram's stain** | ***n*** | **PNA FISH** | ***n*** |
| *C. albicans* | 17 | *C. tropicalis* | 2 | - | |
| | | *C. parapsilosis* | 1 | - | |
| | | Not seen | 1 | - | |
| *C. tropicalis* | 4 | *C. albicans* | 1 | - | |
| *C. glabrata* | 9 | *C. parapsilosis* | 1 | - | |
| *C. krusei* | 4 | *C. parapsilosis* | 3 | - | |
| *C. parapsilosis* | 7 | *C. albicans* | 1 | - | |
| *C. metapsilosis* | 1 | | | *C. albicans/parapsilosis* | 1 |
| *C. orthopsilosis* | 1 | | | *C. albicans/parapsilosis* | 1 |
| *C. guilliermondii* | 2 | *C. glabrata* | 1 | - | |
| *C. lusitaniae* | 1 | *C. glabrata* | 1 | - | |
| *C. pelliculosa* | 1 | *C. glabrata* | 1 | - | |
| *C. neoformans* | 2 | - | | *C. glabrata/krusei* | 1 |
| *B. capitatus* | 1 | - | | *C. glabrata/krusei* | 1 |
| Total | 50 | | 13 | | 4 |
| Mixed blood cultures | | | | | |
| *C. glabrata/tropicalis* | 1 | *C. parapsilosis* | 1 | *C. glabrata/krusei* only | 1 |
| *C. albicans/tropicalis* | 1 | *C. parapsilosis* | 1 | - | |

Results by probe are summarized in Table 6.4. The sensitivity for the *C. albicans/C. parapsilosis* probe was 100% (27/27), for the *C. glabrata/C. krusei* probe was 92.3% (13/14), and for the *C. tropicalis* probe was 100% (6/6). The specificity for the *C. albicans/C. parapsilosis* probe was 100% (27/27), for the *C. glabrata/C. krusei* probe was 95% (38/40), and for the *C. tropicalis* probe was 100% (48/48).

**Table 6.4 PNA FISH results for 52 yeast blood cultures displaying performance for each individual probe**

| | | PNA FISH result | | | |
|---|---|---|---|---|---|
| **Blood cultures** | ***n*** | ***C. albicans* *C. parapsilosis* (green)** | ***C. tropicalis* (yellow)** | ***C. glabrata* *C. krusei* (red)** | **Negative** |
| *Candida albicans* | 17 | 17 | - | - | - |
| *Candida parapsilosis* | 7 | 7 | - | - | - |
| *Candida orthopsilosis* | 1 | 1 | - | - | - |
| *Candida metapsilosis* | 1 | 1 | - | - | - |
| *Candida tropicalis* | 4 | - | 4 | - | - |
| *Candida krusei* | 4 | - | - | 4 | - |
| *Candida glabrata* | 9 | - | - | 9 | - |
| *Candida glabrata/tropicalis* | 1 | - | 1 | - | - |
| *Candida albicans/tropicalis* | 1 | 1 | 1 | - | - |
| *Candida guillermondii* | 2 | - | - | - | 2 |
| *Candida lusitaniae* | 1 | - | - | - | 1 |
| *Candida fabianii* | 1 | - | - | - | 1 |
| *Blastoschizomyces capitatus* | 1 | - | - | 1 | - |
| *Cryptococcus neoformans* | 2 | - | – | 1 | 1 |
| Specificity | | 100% | 100% | 92.9% | 71.4% |
| Sensitivity | | 100% | 100% | 95% | 97.9% |

#### 6.3.3.1 Cost, turnaround time and technical requirement

The reagent cost of PNA FISH YTL was £44.00 per test, exceeding that of Gram's stain at £0.13. When measuring the turnaround time for processing a single blood culture, which would be expected in a routine setting as *Candida* cultures are infrequent, the Gram's stain analysis was more rapid for 15 minutes per sample compared with an average time of 90 minutes for PNA FISH YTL analysis. When assessing the technical requirement a scientist with only 3 days training was able to complete all analyses without any error of further assistance which was considered minimal technical requirement for PNA FISH YTL. In contrast Gram's stain required a considerable amount of experience, four months experience in this study, and even with this experience the performance of this method was not optimal. The inexperienced scientist demonstrated poor performance when identifying yeasts by Gram stain analysis therefore this method couldn't be performed with limited technical experience.

### 6.3.4 Antifungal impact assessment

When analysing the antifungal impact through case note review data suggested that using PNA-FISH a switch from caspofungin to fluconazole could have been made in 21.2% (11/52) cases, and a switch from fluconazole to caspofungin could have been made in 7.7% (4/52) cases, figure 6.2. There would have been no change in antifungal therapy for 61.5% (32/52) of the cases of candidemia. For 5 cases there was insufficient data to indicate which antifungal decision may have been made.

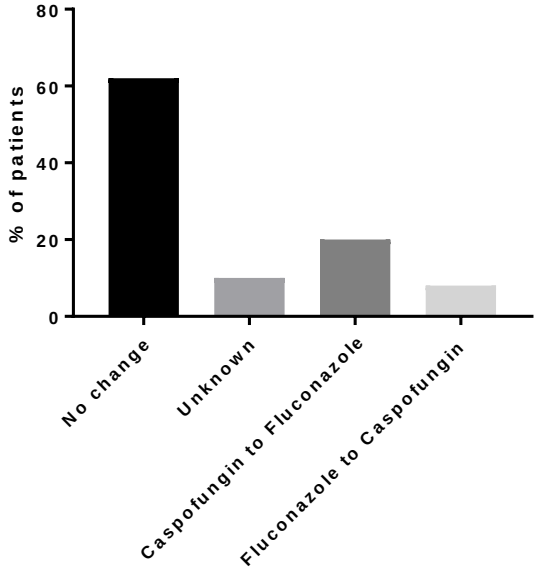



Figure 6.2 Histogram detailing the proportion of clinical decisions that may have been impacted by the PNA FISH result if it had been available to clinicians at the time of diagnosis.

## 6.4 Discussion

The aim of this study was to evaluate the utility of two rapid microscopic techniques for the identification of yeasts direct from blood culture. The method returning the highest number of correct identifications, compared with ITS rRNA sequenced based analysis, was the PNA FISH YTL assay achieving 96% (48/50) success. These findings are supported by two further studies publishing 96% and 99.3% success rates (Hall *et al.*, 2005; Farina *et al.*, 2015) Gram's stain analysis identified 72% (36/50) of the yeasts isolates. Several factors led to the lower success rate of Gram's stain.

Previous work has investigated the use of Gram's stain for the differentiation of *C. albicans* from non-albicans species from blood cultures (Harrington *et al.*, 2007) but to my knowledge this study is the first to investigate the use of yeast morphology in Gram's stain for identification of several *Candida* species. Misidentification was the

primary reason for the lower success rate, with 26% (13/50) of the yeast cultures being misidentified, despite an experienced health care scientist analysing the slides. Both commonly encountered yeasts and rare species were misidentified Only one result of 'unknown' was recorded despite five of the yeast species in this study being absent from the identification keys, indicating a bias to identify rare yeasts as commonly encountered species. This could also be bias against perceived 'failure' to identify by the scientist analysing the slides, in that giving an identification is perceived to be better than not giving one at all (Rousseau *et al.*, 1998; Chancey *et al.*, 2017). A report of 'no yeasts seen' for one blood culture was likely to be an examination error as all blood cultures exceeded the estimated limit of detection (LOD) for Gram's stain of $1x10^5$ cfu/mL (Branda, Ferraro and Kratz, 2007).

The misidentification of fluconazole resistant *C. krusei* isolates as *C. parapsilosis* was noted as of particular concern. This would have had a negative clinical impact assuming that an identification of *C. parapsilosis,* a species commonly fluconazole susceptible, would have influenced a switch in empirical therapy from an echinocandin to fluconazole. However, 83% (14/17) of the *C. albicans* isolates were identified correctly. Three *C. albicans* misidentifications included two as *C. tropicalis* and one as *C. parapsilosis.* Both species are considered to be azole susceptible and a switch to fluconazole would not have been inappropriate in both instances in an uncomplicated patients (Ullmann, *et al.*, 2011). The inclusion of pseudohyphal clustering in the identification keys for *C. albicans* was the likely factor contributing to this higher success rate for this species. This finding is supported by the previous study which also utilised pseudohyphal clusters to identify *C. albicans* from blood cultures demonstrating 97% specificity, with a single false positive result reported as *C. tropicalis* (Harrington *et al.*, 2007). There is a potential role for the use of Gram's stain to differentiate *C. albicans* from other *Candida* species in a centre where an experienced health care scientist is available. This approach could be useful in a low technology diagnostic setting. It should be noted that only a small proportion of other pseudohyphal species were included in this study but may explain the misidentification of *C. albicans* as pseudohyphal forming species *C. tropicalis and C. parapsilosis*.

The success of the PNA FISH YTL assay was attributed to the minimal technical expertise necessary to perform the assay and the high specificity of the PNA probes for their target molecule, exceeding 95% across all published studies (Hall *et al.*, 2005; Heil *et al.*, 2012; Farina *et al.*, 2015; Radic *et al.*, 2016). The highly visible fluorescent signals seen using microscopy enable accurate results interpretation and only require a

limited amount of training and hands on time to be competent in interpreting results. Non-specific fluorescence or cross-reaction of the PNA probes with human blood components was not observed. Overall 86% (43/50) of the yeasts included in this study were identified. The species distribution included in this study does not reflect the normal distribution experienced in the clinical setting. Greater than 95% of fungaemia is attributed to the 5 yeast species contained within the assays database (Odds *et al.*, 2007; Ullmann, *et al.*, 2011) and 100% (43/43) of the species contained within the assays database were identified correctly. Using the PNA FISH YTL assay routinely only a small proportion of yeasts implicated in Candidemia would require an additional 24 hours before identification is achieved. Two misidentifications occurred using the PNA FISH YTL assay, one *C. neoformans* and one *M. capitatus* isolate, both cross reacting with the red *C. krusei/C. glabrata* probe.

Assuming the respective *C*. *glabrata*/*C. krusei* results would have prompted the administration of an echinocandin, in both instances an echinocandin would have been an inappropriate therapy choice. Until phenotypic identification had been achieved 24-72 hours later the fungal infections would have remained untreated. Although *C. neoformans* fungaemia is encountered infrequently in the laboratory, the unique morphology of this yeast in Gram's stain often provides an indication of Cryptococcaemia. If this had been detected prior to performing PNA FISH YTL it could have prevented this misidentification from occurring, therefore in the routine laboratory setting this misidentification may have been avoided. *M. capitatus* is rarely encountered in fungaemia and should have been reported as negative by the PNA FISH assay. It is likely that a negative PNA FISH YTL result would have prompted the use of an echinocandin as empirical therapy until the yeast identification was achieved from culture, thus the treatment course would have remained unchanged. When repeated both analyses were reported as negative, which may indicate insufficient washing of the slide during analysis to be the likely cause of the false positive misidentifications or background debris reported as fluorescence. One other study reported misidentifications with the *C. glabrata/C. krusei* PNA FISH YTL probe cross reacting with the rarely encountered yeast *Candida bracarensis* (Hall *et al.*, 2012).

This study has shown that Gram's stain analysis is inexpensive and rapid. However, Gram's stain did not achieve the required diagnostic performance for yeast identification to species level. It was able to divide *Candida* species into *C. albicans* and species other than *C. albicans* which could in turn be used to drive the choice of antifungal therapy as *Candida abicans* are in the majority of isolates fully susceptible to

azoles (Castanheira *et al.*, 2016). The PNA FISH YTL assay is rapid, successful and requires limited technical expertise but may be considered expensive at a list price of £44.00 per analysis. The initial cost could be offset by influencing a switch in antifungal therapy from an echinocandin to fluconazole in a proportion of patients. For yeasts included in this study a retrospective analysis of antifungal therapy at the time of fungaemia estimated a change in antifungal therapy could have been made in 29% of patients. This was based on a retrospective review of clinical notes, which as an approach has its limitations, as records are often not complete. Clinical records of when or which antifungal was administered were not always clear, and the information for some patients was not available. Additionally, the number of isolates tested was small, particularly those of species that the assay states it is unable to identify ($n$ =7). However, a recent prospective study conducted in the USA calculated cost savings of $415 per patient through direct influence on targeted antifungal therapy using the results of the YTL assay (Heil *et al.*, 2012). A significant decrease in time to targeted therapy and higher rate of culture clearance was also demonstrated (Heil *et al.*, 2012). With the frequency of blood cultures growing yeasts remaining low, in our 900-bed hospital we experience 12-20 cases of fungemia per year (BSMM, 2003), the financial burden of the YTL PNA FISH assay is not substantial and could ultimately lead to financial savings (and improved clinical outcome) if clinical management can be influenced by the diagnostic results.

In summary, Gram's stain analysis of blood cultures will remain essential in the laboratory as a first line test to determine the presence of yeasts within a blood culture, and as shown in this study may serve as a means of discriminating non-albicans species in a low technology environment. This study has demonstrated that the PNA FISH YTL assay is highly sensitive and specific, with a short turnaround time. Favourable outcomes in prospective, randomized trials of clinical utility could lead to more widespread adoption of this assay in routine clinical laboratories.

# Chapter 7 Evaluation of a second generation PNA-FISH assay, *Quick*FISH™, for the rapid identification of yeasts direct from positive blood cultures.

## 7.1 Overview

Peptide nucleic acid fluorescence *In situ* hybridisation (PNA-FISH) has demonstrated a high degree of accuracy, 100% (43/43), for the identification of yeasts direct from blood culture films (chapter 6). The processing of PNA-FISH YTL slides had a turnaround time of 90 minutes for one blood culture. The World Health Organisation (WHO, 2017) considers a 'rapid' test as a test that provides a result within 10 minutes – 2 hours (WHO, 2017). Therefore, the YTL PNA-FISH assay can be classified as a rapid test according to these criteria. However, for the PNA-FISH result to be effective, clinicians would have to wait 90 minutes to report out the result, withholding the grams stain result in the interim. This is a difficult position clinically as studies have shown candidemia mortality can increase hour on hour and guidelines recommend the earliest possible opportunity for intervention should be taken to administer antifungal therapy (Fraser *et al.*, 1992; Alonso-Valle *et al.*, 2003; Colombo *et al.*, 2007). For PNA-FISH to truly meet the rapid test criteria and for the result to be actionable the result needs to be available as close as possible to the time the Gram's stain result is available

In 2012 a second-generation PNA FISH assay, *Quick*FISH™ (AdvanDX, Woburn, MA, USA), was developed for routine diagnostic use. The PNA-FISH assay processing was reformatted, removing the lengthy hybridisation and slide washing steps in the protocol, into a simple two-step approach incorporating a fixation step, probe application to the fixed blood smear and a final 15 minute shortened hybridisation, figure 7.1. The removal of the wash buffer step in the process shortened the overall protocol turnaround time to approximately 20 minutes. The reduction in turnaround time was achieved by removing the need to stringently wash residual unbound probe by reformatting the assay to contain quencher-probe complexes, replacing the old format of 'free' PNA probe. The quencher molecules complex with the PNA probe as the slide cools to room temperature. When added to the slide and hybridised at 56ºC the quencher dis-associates from the PNA probe allowing the PNA probe to bind to its target. After hybridisation the slide cools to room temperature and any unbound probe re-forms a complex with its quencher molecule. The quencher molecules neutralize the fluorescence emitted from unbound probes and only fluorescence from PNA probes bound to their target in-situ can be visualized. The manufacturers also modified the

fixation step steps to include two fixative solutions that are applied to the blood culture smear prior to probe hybridisation. The modified dual fixative improves the permeability of the yeast cell wall to the PNA FISH probes allowing for more rapid and efficient in-situ hybridisation, shortening hybridisation from 30 to 16 minutes at 56°C.

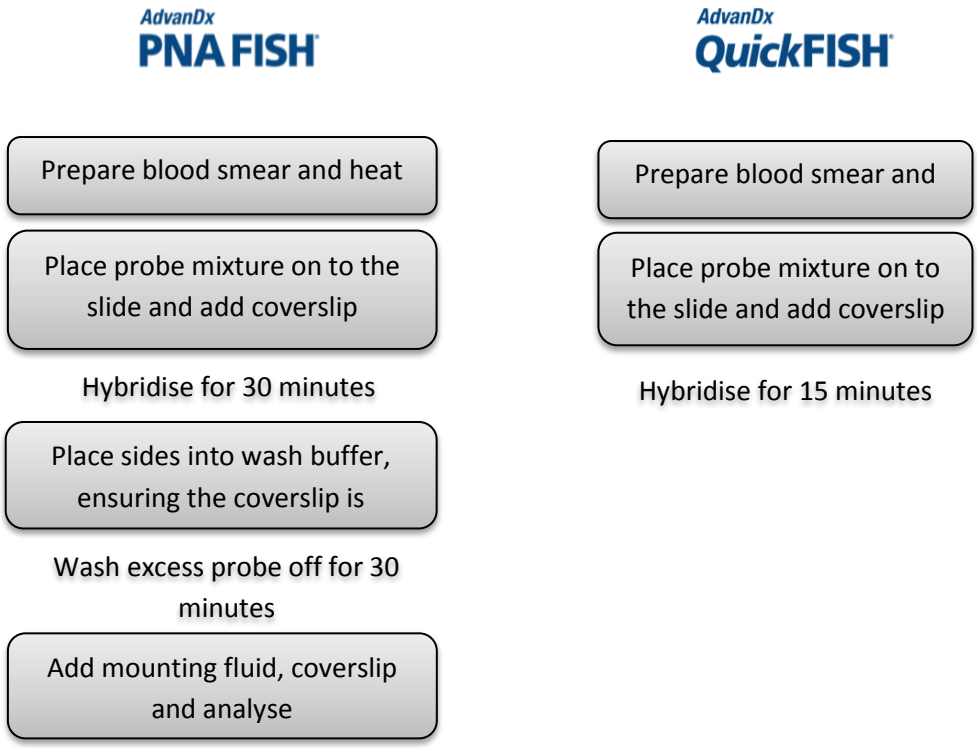



Figure 7.1.Comparison of PNA-FISH and *Quick*FISH processing methods.

An additional change was made to the *Candida Quick*FISH™ assay (AdvanDX, Denmark) in that the assay was reformatted to exclude *C. tropicalis* and *C. krusei* as targets with a streamlined format targeting only three species; *C. albicans* (green), *C. parapsilosis* (yellow) and *C. glabrata* (red). The decision to reformat the assay format was based on the prevalence of each species in candidemia. In the USA and Europe *C. albicans* accounts for >50% of candidemia and C. glabrata has emerged as an important pathogen (Wisplinghoff *et al.*, 2014; Kullberg and Arendrup, 2015). *C. glabrata* is the second most prevalent species accounting for 10-20% (Odds *et al.*, 2007; Ruan *et al.*, 2008; Kett *et al.*, 2011; Silva *et al.*, 2012; Moretti *et al.*, 2013; Deorukhkar, Saini and Mathew, 2014) and finally *C. parapsilosis*, known to cause candidemia in premature neonates accounts for 33.4% of candidemia in the neonatal

patient cohort (Singh and Parija, 2012; Pammi *et al.*, 2013) but also 12% in adults (Odds *et al.*, 2007). The impact of removing *C. tropicalis* and *C. krusei* from the kit format may be significant as *C. tropicalis* is implicated in 3-66% of candidemia, frequency varying with geography (Ann Chai, Denning and Warn, 2010) and is known to be more virulent than other *Candida* species, especially in the haemato-oncology setting (Kontoyiannis *et al.*, 2001). *C. krusei* accounts for 2.1% of candidemia (Pfaller *et al.*, 2011), therefore not identifying this species may be of less concern. However, *C. krusei* is intrinsically resistant to fluconazole, used as prophylaxis and as first-line therapy in some patients.

The aim of this study was to evaluate the performance of *Quick*FISH for the identification of yeasts direct from positive blood cultures. A retrospective audit of fungemia was performed to establish the proportion of yeasts isolated in our setting that would have been identified by the new assay format and to assess the impact that removing *C. tropical* and *C. krusei* would have had on the diagnosis of Candidemia in our hospital.

## 7.2 Methods

### 7.2.1 Yeast isolates

Eighty-one yeast isolates were selected from a clinical archive of isolates retained at the Royal Free Hospital microbiology laboratory. Isolates were selected to represent a diverse collection of species encountered in fungemia. All isolates were from previous cases of fungemia from patients treated at the Royal Free Hospital. Isolates were stored in sterile water; away from light at room temperature. Throughout the study all prospective blood cultures positive for yeasts by Gram's stain were also included for analysis with *Quick*FISH.

### 7.2.2 Subculture of yeast isolates

See general methods section 2.1.1

### 7.2.3 Simulated blood cultures

See general methods section 2.1.2

### 7.2.4 Routine blood culture investigation

Blood smears were prepared from 1 drop of culture and the smears were fixed on a heated slide station. Once dried a conventional Gram's stain method was applied. For subculture 2-3 drops of positive blood culture was inoculated onto blood Columbia, chocolate and cystine lactose electrolyte deficient agars (Oxoid Wesel, Germany) with the addition of a blood agar plate incubated anaerobically. All subcultures were incubated at 36°C for 18-24 hours.

### 7.2.5 MALDI-TOF identification of yeasts

See general methods section 2.3.1 - 2.3.3

### 7.2.6 ITS rRNA sequenced based identification of yeasts

See general methods 2.2.

### 7.2.7 Preparation of *Quick*FISH slides

All work was performed in a class 2 microbiology safety cabinet. *Quick*FISH slides were pre-warmed for 1 minute on a slide station at 55 ± 1°C. Using a vacutainer blood collection device and syringe 0.5mL of blood culture was transferred to a microfuge tube. 10µL of the blood culture was pipetted from the microfuge tube to the centre of the *Quick*FISH slide sample area. One drop of *Quick*Fix-1 was immediately added to the sample, and the mixture was spread evenly throughout the sample area on the slide with an inoculating needle, see figure 7.2. The slide was transferred back to the slide station and heat fixed until the smear was visibly dried (1–3 minutes). Two drops of *Quick*Fix-2 were added to the centre of the sample area and allowed to dry (1 minute). One drop of PNA Blue was applied to the centre of a 24 × 50mm coverslip, followed by 1 drop of PNA Yellow. The blue and yellow hybridization reagents were thoroughly mixed with a mixing stick until a uniform green colour was observed (approximately 5–10 seconds). Coverslips were inverted and applied to the *Quick*FISH slides to cover the control wells and the fixed samples with the hybridization reagent mixture. Slides were then incubated on a heat block at 55 ± 1°C for 15 minutes.

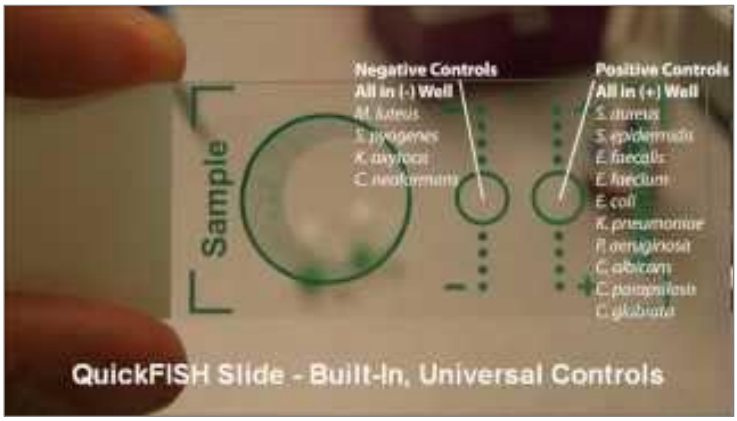



Figure 7.2 *Quick*FISH slide demonstrating sample preparation area, negative and positive control areas on the pre-prepared slide.

### 7.2.8 Microscopic analysis of *Quick*FISH slides

Slides were analysed using an Olympus BX63 fluorescence microscope (objective, 100×, Olympus BX40; Osram HBO 100 W/2 Hg lamp) equipped with a fluorescein isothiocyanate-Texas Red double filter (filter no. AC003, AdvanDx). Slides were analysed from the positive control field to ensure hybridisation was successful for all yeasts and the negative control (containing a smear of *Cryptococcus* cells) to ensure cross hybridisation did not occurred. The sample section was analysed last for results interpretation. All areas of the sample field were examined to ensure mixed yeast cultures were not present. Figure 7.3 demonstrates typical fluorescence seen when analysing *Quick*FISH *Candida* slides with C. albicans (green), C. parapsilosis (yellow) and C. glabrata (red) demonstrated. A mixed culture representing a typical image of the positive control is also shown.

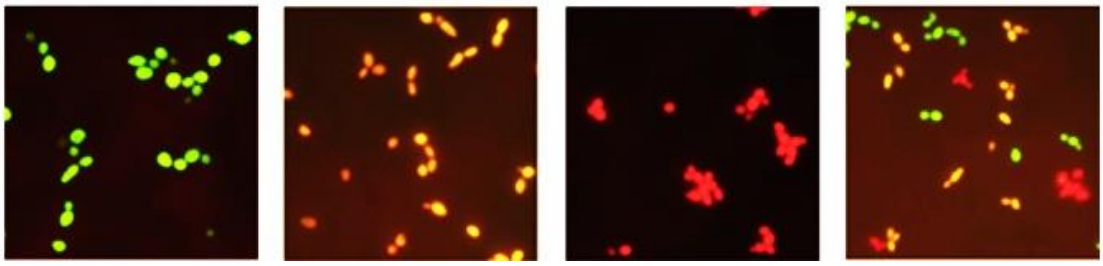


Figure 7.3 Fluorescence signal observed when analysing *Quick*FISH *Candida* slides with *C. albicans* (green), *C. parapsilosis* (yellow) and C. glabrata (red) demonstrated. A mixed culture representing a typical image of the positive control is also shown.

### 7.2.9 Fungaemia audit

A twelve-month retrospective audit, from 1st January 2013 to 31st December 2013, of fungaemia episodes documented at our centre was performed. The aim was to establish the prevalence of yeast species implicated in fungaemia and the potential performance of the *Quick*FISH assay in our setting. The yeast identification reported out from the blood culture was collected on our local WinPath reporting system.

## 7.3 Results

### 7.3.1 Yeast blood cultures

In total 96 blood cultures were included in the study. Of the 96 blood cultures, 15 were tested in real-time from cases of fungaemia and 81 were spiked blood cultures. Spiked yeast cultures included yeasts from 3 genera and 19 species, of which 65.9% (54/82) of the isolates were contained within the *Quick*FISH assay format. A full break down of the species distribution is presented in table 7.1.

**Table 7.1 Species distribution for yeasts from 96 blood cultures analysed using *Quick*FISH in this study**

| **Species** | **Spiked** | **Clinical** | **Total** | |
|---|---|---|---|---|
| | *n* | *n* | % | *n* |
| *Candida albicans* | 33 | 11 | 45.8 | 44 |
| *Candida glabrata* | 14 | 2 | 16.7 | 16 |
| *Candida parapsilosis* | 7 | | 7.3 | 7 |
| *Candida krusei* | 5 | | 5.2 | 5 |
| *Candida tropicalis* | 3 | | 3.1 | 3 |
| *Candida dubliniensis* | 2 | | 2.1 | 2 |
| *Candida guillermondii* | 2 | | 2.1 | 2 |
| *Candida kefyr* | 2 | | 2.1 | 2 |
| *Pichia cactophila* | 2 | | 2.1 | 2 |
| *Candida sp* | - | 2 | 2.1 | 2 |
| *Candida famata* | 1 | | 1.0 | 1 |
| *Candida lambica* | 1 | | 1.0 | 1 |
| *Candida utilis* | 1 | | 1.0 | 1 |
| *Candida lusitaniae* | 1 | | 1.0 | 1 |
| *Candida metapsilosis* | 1 | | 1.0 | 1 |
| *Candida bracariensis* | 1 | | 1.0 | 1 |
| *Trichosporon inkin* | 1 | | 1.0 | 1 |
| *Trichosporon mucoides* | 1 | | 1.0 | 1 |
| *Cryptoccus neoformans* | 1 | | 1.0 | 1 |
| *Saccharomyces cerevisiae* | 1 | | 1.0 | 1 |
| *Candida fermentati* | 1 | | 1.0 | 1 |
| **Total** | **81** | **15** | **100** | **96** |

### 7.3.2 Performance of *Quick*FISH

Table 7.2 details the performance characteristics of *Quick*FISH, overall and by probe set. Overall, *Quick*FISH demonstrated 98.5% (93/96, CI: 91.2, 98.9) diagnostic accuracy when compared against the conventional identification method MALDI-TOF MS. Sensitivity was 98.5% (66/67 CI: 92.0, 99.7), specificity was 93% (27/29 CI: 78.0, 98.1), PPV was 97.1% (66/68 CI: 89.9, 99.2) and NPV was 96.4% (27/28 CI: 82.3, 99.4).

#### 7.3.2.1 *C. albicans* probe performance

The green *Quick*FISH *C. albicans* probe demonstrated 100% (96/96, CI: 96.2, 100) diagnostic accuracy when compared against the conventional identification method. The sensitivity of the *C. albicans* probe was 100% (44/44, CI: 91.8, 100), specificity of 100% (52/52, CI: 93.2, 100), PPV of 100% (44/44, CI: 91.8, 100) and NPV of 100% (52/52, CI: 93.2, 100). No incorrect results were recorded for the *C. albicans* PNA probe.

#### 7.3.2.2 C. parapsilosis probe performance

The *Quick*FISH yellow *C. parapsilosis* probe demonstrated 100% (96/96, CI: 96.2, 100) diagnostic accuracy when compared against the conventional identification method. The sensitivity of the *C. parapsilosis* probe was 100% (7/7, CI: 64.6, 100), specificity of 100% (89/89, CI: 95.6, 100), PPV of 100% (7/7, CI: 64.6, 100) and NPV of 100% (89/89, CI: 95.6, 100). No incorrect results were recorded for the *C. parapsilosis* PNA probe.

#### 7.3.2.3 C. glabrata probe performance

The *Quick*FISH red *C. glabrata* probe demonstrated an overall diagnostic accuracy of 96.9% (93/96, CI: 91.2, 98.9) when compared to the conventional identification method. The sensitivity of the *C. glabrata* probe was 93.8% (15/16, CI: 71.7, 98.9), specificity of 97.5% (78/80, CI: (91.3, 99.3), PPV of 88.2% (15/17 CI: 65.7, 96.7) and NPV of 98.7% (78/80, CI: 93.2, 99.8). Three incorrect results were recorded using the red *C. glabrata* probe. One *C. glabrata* isolate was missed on *Quick*FISH analysis with a report of ‘no colour’ recorded. Two blood cultures containing yeasts other than *C. glabrata* were reported as positive with red fluorescence, table 7.3.

#### 7.3.2.4 Performance for the identification of other Yeasts

For yeasts not targeted by the *Quick*FISH *Candida* probes an overall diagnostic accuracy of 96.9% (93/96, CI: 93.9, 99.8) was observed when compared to the conventional identification method if a result of no colour was considered as the correct result. Sensitivity was 92% (23/25, CI: 75.0, 97.8), specificity 98.6% (70/71, CI 94.4, 100), PPV 95.8% (23/24, CI: 79.8, 99.4) and NPV 97.2% (70/72, CI: 90.4, 99.2).

### 7.3.3 Assessing assay cost and turn-around-time

Reagent costs of the *Quick*FISH assay were £19.50 per test not including ancillary consumables): calculated by dividing the cost of the kit by the total number of tests per kit. The average turnaround time, measured across the 15 prospective analyses during the course of the study, was 21 minutes (range 19-21) per slide for processing and 23.2 minutes (range 19.8-27.4) per slide including analysis.

**Table 7.3 Isolates misidentified or unidentified from blood cultures *Quick*FISH compared with the expected result.**

| | | **Misidentifications** | | | |
|---|---|---|---|---|---|
| **Isolate** | ***n*** | **Expected result** | ***n*** | ***Quick*FISH** | ***n*** |
| *C. glabrata* | 1 | Red fluorescence | 1 | Negative | 1 |
| *C. braciariensis* | 1 | Negative | 1 | Red fluorescence | 1 |
| *Candida sp.* | 1 | Negative | 1 | Red fluorescence | 1 |

**Table 7.2. Performance characteristics of *Quick*FISH for the identification of yeasts direct from 96 blood cultures, overall performance and performance by probe set is represented.**

| | **Diagnostic accuracy** | | | | **Sensitivity** | | | | **Specificity** | | | | **PPV** | | | | **NPV** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **%** | **95% CI** | | ***n*** | **%** | **95% CI** | | ***n*** | **%** | **95% CI** | | ***n*** | **%** | **95% CI** | | ***n*** | **%** | **95% CI** | | ***n*** |
| Overall | 98.5 | 91.2 | 98.9 | 93/96 | 98.5 | 92 | 99.7 | 66/67 | 93 | 78 | 98.1 | 27/29 | 97.1 | 89.9 | 99.2 | 66/68 | 96.4 | 82.3 | 99.4 | 27/28 |
| *C. albicans* | 100 | 96.2 | 100 | 96/96 | 100 | 91.8 | 100 | 44/44 | 100 | 93.2 | 100 | 52/52 | 100 | 91.8 | 100 | 44/44 | 100 | 932. | 100 | 52/52 |
| *C. parapsilosis* | 100 | 96.2 | 100 | 96/96 | 100 | 64.6 | 100 | 7/7 | 100 | 95.6 | 100 | 89/89 | 100 | 64.6 | 100 | 7/7 | 100 | 95.6 | 100 | 89/89 |
| *C. glabrata* | 96.9 | 91.2 | 98.9 | 93/96 | 93.8 | 71.7 | 98.9 | 15/16 | 97.5 | 91.3 | 99.3 | 78/80 | 88.2 | 65.7 | 96.7 | 15/17 | 98.7 | 93.2 | 99.8 | 78/80 |
| Other yeasts | 96.9 | 93.9 | 99.8 | 93/96 | 92 | 75 | 97.8 | 23/25 | 98.6 | 94.4 | 100 | 70/71 | 95.8 | 79.8 | 99.4 | 23/24 | 97.2 | 90.4 | 99.2 | 70/72 |

### 7.3.4 Fungaemia audit

Data extracted from the laboratory reporting system (WinPath, Clinisys Group, UK) from a 12-month time period of fungaemia from 1st January – 31st of December 2013 recorded 38 events of yeasts isolated from blood cultures. The blood cultures were taken from 23 patients. Figure 7.4 details the frequency of the 10 yeast species isolated. *Quick*FISH would have identified 57.9% (22/38) of the yeast isolates from blood culture. 'Other' species included; *Candida lusitaniae* (1), *Trichosporon sp* (1), *Candida famata* (1) and *Candida guillermondii.*

If the PNA-FISH assay format had been available a further 13.2% (5/38) of yeasts from blood culture, all *C. tropicalis* isolates, would have been identified by *Quick*FISH with a total identification rate of 71.1% (27/38), figure 7.4. This difference in the identification rate is not significant when compared with the identification rate that would have been obtained using the current *Quick*FISH assay format (p=0.23).

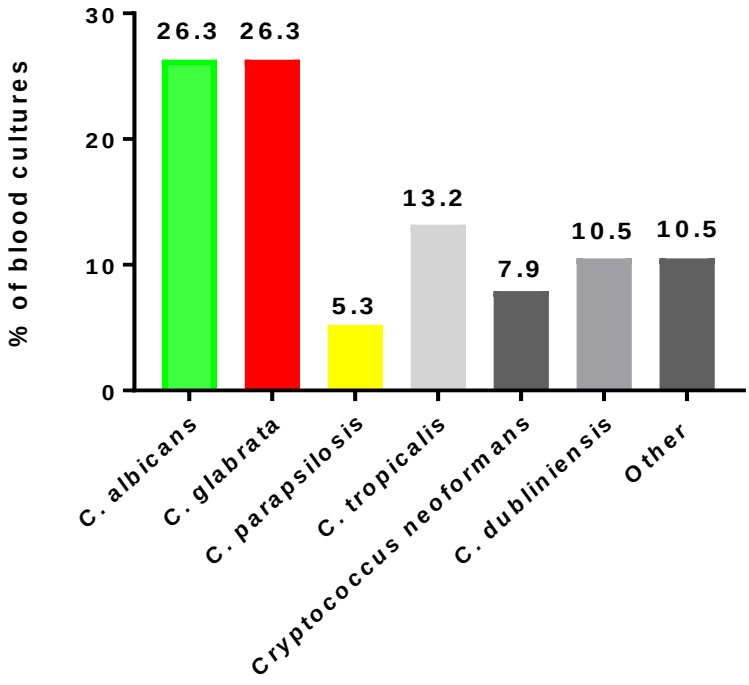



Figure 7.4 Histogram displaying the frequency of yeast species isolated from 38 blood cultures at the Royal Free Hospital microbiology laboratory over a twelve-month period from 1st January – 31st December 2013. The colours demonstrate the colour of the *Quick*FISH probe fluorophores that targets each species.

## 7.4 Discussion

*Quick*FISH is the only nucleic-acid based method to date capable of providing a species identification in less than 30 minutes direct from a positive blood culture, as other approaches such as real-time PCR and DNA microarray require a turnaround time of approximately 2 hours for results (Deck *et al.*, 2014). *Quick*FISH has shown to be a rapid, sensitive and specific method, identifying 98.5% (66/67) of the target species correctly in this study. This high performance of *Candida Quick*FISH is supported by a multicentre trial, which reported a diagnostic accuracy of 99.3% for the identification of the species contained within the assay format, supporting the findings of our study (Abdelhamed *et al.*, 2015).

As *Quick*FISH was launched in 2012 only a small number of reports have been published utilising the assay for the identification of microorganisms from blood culture, but all report high sensitivities and specificities (Deck *et al.*, 2012, 2014; Carretto *et al.*, 2013; Martinez *et al.*, 2014; Salimnia *et al.*, 2014; Abdelhamed *et al.*, 2015; Koncelik and Hernandez, 2016). A trial utilising *Quick*FISH panels for the identification of Enterococci, Gram negative bacilli, Staphylococci and *Candida* reported an overall concordance of 98% with conventional identification (Martinez *et al.*, 2014). In the respective study four yeasts were isolated from blood culture and 100% concordance was observed between *Quick*FISH *Candida* and conventional methods of identification (Martinez *et al.*, 2014). In a multicentre study Deck *et al* investigated the Enterococcus *Quick*FISH panel reporting sensitivities of 100% (106) and 97% (65/67) were recorded for the green (*Enterococcus faecalis*) and red PNA (other enterococci) probes respectively (Deck *et al.*, 2012, 2014). By far the most investigated *Quick*FISH panel to date is the Staphylococcal panel that determines *S. aureus* (green probe) from coagulase negative staphylococci. A multicentre study, investigating the Staphylococcal *Quick*FISH panel performed by Deck *et al* reported sensitivities of 99.5% (217/218) for the green (*S. aureus*) and 98.8% (487/493) for the red (coagulase negative staphylococci) probes (Deck *et al.*, 2012). The success with the Staphylococcus *Quick*FISH kit was also replicated in a further study reporting 100% sensitivity and specificity for the identification of *S. aureus* and a sensitivity of 98.5% and specificity of 100% for coagulase negative staphylococci identification (Carretto *et al.*, 2013). The successful use of the Staphylococcal *Quick*FISH was further supported in a large multicentre trial reporting concordance with the routine phenotypic testing methods of the testing laboratories in 99.1% (1,211/1,221) samples across 5 laboratories (Salimnia *et al.*, 2014) In the respective studies the performance of

*Quick*FISH was slightly lower with the red PNA probe. This was also an observation in our study.

Three errors were reported using the red PNA probe. Two isolates, *C. bracariensis* and *C. nivariensis*, were reported as *C. glabrata* with red fluorescence and one *C. glabrata* was not detected in an analysis. Repeat interrogation of the slide from which C*. glabrata* was missed demonstrated a low quantity of red fluorescent yeast cells amongst the background of non-specific (yellow/orange) auto fluorescence from erythrocytes within the preparation. In this instance the PNA probe had bound to its target but due to a low concentration of yeasts within the blood culture the investigator had failed to detect the yeasts. In part, this is a limitation of utilising a red fluorescent probe as non-specific auto fluorescence from erythrocytes may in some instances mask the specific fluorescence of the bound PNA probe when the quantity of organisms is low in a blood culture. Higher than expected levels of background auto fluorescence has also been attributed to misidentifications made in a similar study evaluation of *Quick*FISH and was associated with the specification of microscope being used for analysis (Martinez *et al.*, 2014). This is an important consideration for laboratories choosing to implement PNA FISH methods as the quality of the microscope, light source; filter wavelength specifications may impact on the accuracy of results (Martinez *et al.*, 2014). Operator variation and error is also a recognised limitation in laboratory based microscopy screening and was concluded to be the cause of two false negative results reported in a staphylococcal *Quick*FISH study (Deck *et al.*, 2012).

Two other discrepancies recorded in our study were related to cross reaction of the *C. glabrata* PNA probe with two genotypically closely related species *C. nivariensis* and *C. bracariensis*. Although these misidentifications have low clinical significance, in our setting it would prompt the use of an echinocandin as both *C. nivariensis* and *C. bracariensis* also have the same inducible mechanism of resistance to fluconazole as *C. glabrata*. Unfortunately the multi-center trial evaluating the *Candida Quick*FISH assay used biochemical profiling for the identification of yeasts. Both *C. nivariensis* and *C. bracariensis* are indistinguishable from *C. glabrata* biochemically so any cross-reaction of the red PNA probe with these cryptic species could not be determined (Lockhart *et al.*, 2009). Investigators in the respective study reported cross-reaction of the *C. parapsilosis* probe with two isolates, *C. metapsilosis* and *C. tropicalis,* (Abdelhamed *et al.*, 2015). The assay manufacturer acknowledges cross-reaction of the *C. glabrata* PNA probe with *C. nivariensis* and *C. bracariensis* and the *C. parapsilosis* probe with *C. orthopsilosis* and *C. metapsilosis* in the product insert. None

of the known cross-reacting misidentifications are considered to be clinically significant in our setting, as they would not impact on the therapeutic management of patients. What is of some concern is the limited range of species that the *Candida Quick*FISH assay can identify and the uncertainty when a negative result is reported by the assay.

The three species included in the assay format do represent the three commonest *Candida* species implicated in fungaemia, reportedly responsible for 74%-88.2% of the burden of disease (Chalmers *et al.*, 2011; M. A. Pfaller *et al.*, 2011; Orasch *et al.*, 2014; Trouvé *et al.*, 2017). As a second-generation assay, the manufacturers determined *Quick*FISH should not include *C. tropicalis* and *C. krusei* PNA probes in the assay assay format. Whilst *C. krusei* does not represent a significant proportion of the fungaemia burden globally, *C. tropicalis* is commonly isolated from blood cultures (M. A. Pfaller *et al.*, 2011) and accounted for a significant proportion of the blood cultures, 13.2% (5/38), encountered in our setting. The removal of the *C. tropicalis* PNA probe has had an impact on the overall specificity of the assay. An audit of *Candida* blood cultures processed over a twelve month period from 1st January 2013 to 31st December 2013 revealed that only 59.5% (22/37) of yeasts would have been identified. The addition of a *C. tropicalis* probe would have increased success to 73% (27/37) in our setting. In the multicentre trial investigating the *Candida Quick*FISH assay 26% of yeasts were not identified (8.8% of the isolates being *C. tropicalis)* (Abdelhamed *et al.*, 2015).

The SENTRY antimicrobial surveillance program published data from North and South America. *C. tropicalis* was the fourth most common cause of fungaemia representing 11% of the burden (Pfaller *et al.*, 2011). In an Indian tertiary care hospital in the haemato-oncology setting *C. tropicalis* was the most common cause of fungaemia accounting for 46.7% of the isolates encountered in blood cultures (Kaur *et al.*, 2017). The inclusion of a *C. tropicalis* probe would only have a positive impact on assay performance and would benefit in selecting a global population of *Candida* species, suiting a broader array of healthcare environments. The decision to omit the number of probes in the assay format for *Candida* is most likely not based on technical limitations but more related to the regulatory claims required to produce an *in vitro* diagnostic assay. The *S. aureus* panel and the Enterococcus panel has a broad range of species included within the assay format. The Staphylococcal *Quick*FISH assay has been shown to detect 28 species of coagulase negative staphylococci in the red probe mix (AdvandDX manufacturer's validation) and the enterococci assay detects 14 non-faecalis enterococci species (AdvandDX manufacturers validation). PNA as a technique can incorporate multiplexed probes within a single mix without impacting on

the performance of the test. If differing probe combinations were available for centres with a more diverse range of yeast species implicated in fungaemia, where specialist patient groups have a reduced incidence of candidemia with the more common species contained within the *Candida* *Quick*FISH format, this would be of greater benefit to a broader range of clinical services. The inability of *Quick*FISH to identify 40% of yeasts from blood cultures in the prospective audit in our study is not adequate for the diagnostic service. However, it is also important to acknowledge that a negative result is informative as it indicates the presence of a less common species.

The clinical impact of *Quick*FISH is yet to be firmly established. In a prospective cohort study of patients receiving antifungal therapy for candidemia it was estimated that using the first generation PNA FISH *Candida* assay antifungals could have been initiated 24 hours earlier compared with standard microbiological practice for yeast identification (Aitken *et al.*, 2014). As the mortality of Candidemia is known to increase dramatically over 24 hours, earlier initiation of antifungal therapy could lead to decreased mortality for patients (Garey *et al.*, 2006). In an impact study investigating the Staphylococcal *Quick*FISH assay the average length of stay and days on vancomycin were all decreased significantly ($P = .0484$, and $P = .0084$, respectively) as the result of *Quick*FISH testing; for acute-care patients hospitalized for 10 days or less (Koncelik and Hernandez, 2016). This indicates that *Quick*FISH can have a positive impact downstream on outcomes from blood stream infection. As with any new test it is important not only to establish clinical impact but also implement an appropriate testing rationale, establishing local guidelines to ensure that the test is used in the correct setting. In our laboratory an algorithm was developed to screen positive blood cultures and only new positive cultures for a patient that had not had a previous blood culture tested within a 72 hour window. A similar decision-making strategy was also implemented by Almangour *et al* who developed the strategy for rapid testing, only performing *Quick*FISH in patients where the test would have an impact if a staphylococcal isolate was encountered or in a patient whereby a foreign device was *In situ* or was not being treatment with vancomycin. This was a slightly different approach to diagnostic implementation, the driver being clinical as opposed to laboratory operational. However, this approach maintained a reduction in the number of tests from a possible 43 blood cultures to 9, equating to an approximate saving of £3000 in reagent costs (Almangour, Alhifany and Tabb, 2017) This highlights the optimal implementation strategy of rapid screening tests requires a combined clinical and laboratory approach to obtain the best testing strategy.

In summary, this study has demonstrated that the *Candida Quick*FISH assay is an accurate and rapid test for the identification of yeasts within minutes from positive blood cultures and may contribute to targeted clinical management, antimicrobial stewardship and improved patient care whilst benefiting via an improved healthcare economic model. Earlier identification has also been associated with decreased costs with a delay in antifungal therapy increasing the attributed hospital costs by approximately £8000 (Arnold *et al.*, 2010; Taur *et al.*, 2010; Zilberberg *et al.*, 2010). Future work should include trials of the assay to ascertain its clinical utility so that the additional cost per test added to a blood culture of approximately £20 can be justified.

# Chapter 8 Evaluating the performance of *P. jirovecii* PCR alongside automated DNA extraction; a comparison of the MycAssay/Diasorin IXT and the BD MAX™

## 8.1 Introduction

Many laboratories still rely on cytological staining methods to detect and diagnose *P. jirovecii* infection from brochoalveolar lavage (BAL) and sputum specimens (Schelenz *et al.*, 2009). The sensitivity of microscopy is known to be suboptimal. Grocott's staining; a methenamine silver stain has reported sensitivities of 50-89.7% and immunofluorescence 60-93.1% (Flori, 2004; Procop *et al.*, 2004; Alanio, Desoubeaux, *et al.*, 2011). In addition microscopy cannot be used to exclude disease when a test is negative. In most patient cohorts the incidence of PCP is relatively low (Kim *et al.*, 2013; Sarwar *et al.*, 2013; Kostakis, Sotiropoulos and Kouraklis, 2014), in this scenario the diagnostic test is used to rule out disease with a high negative predictive value and a high sensitivity for detecting *P. jirovecii* from specimens (White *et al.*, 2017). This in turn can result in false positive results as colonising *P. jirovecii* will be defected (White *et al.*, 2017) but positive results can be assessed in the context of the patient and the clinical presentation to assess the significance of the result. In a low prevalence setting microscopy should not be the primary method of diagnosis as PCP, but rather a more sensitive assay such as PCR is better suited (White *et al.*, 2017).

The use of PCR for the detection of *P. jirovecii* infection is not a novel approach, with many studies detailing the use of PCR for the diagnosis of PCP for over 20 years. In three recent meta-analyses the performance of PCR was shown to be highly sensitive ranging from 97-99% and the negative predictive value was very high ≥99% for the exclusion of PCP (Lu *et al.*, 2011; Fan *et al.*, 2013; Summah *et al.*, 2013). In the respective meta-analyses the specificity for the detection of *P. jirovecii* ranged from 90-94% with a positive predictive value of 66-85% reflecting the complexities of distinguishing infection from colonisation when utilising PCR (Lu *et al.*, 2011; Fan *et al.*, 2013; Summah *et al.*, 2013). The diagnosis of PCP using PCR is not without challenges and most likely explains why some clinical services choose microscopy as their method of choice as the limit of detection is lower (poorer) therefore colonisation is not detected (Armbruster, Pokieser and Hassl, 1994; Flori *et al.*, 2004; Seah *et al.*, 2012a; Moodley, Tempia and Frean, 2017).

When implementing a PCR based methodology for the diagnosis of PCP the workflow of the specimen and its processing are crucial in determining the performance of the test. Some diagnostic centres choose to target whole cell DNA by centrifugation of the specimen and subsequent extraction from the resulting concentrate (Flori, 2004; Tia *et al.*, 2012; Matsumura *et al.*, 2014; Robert-Gangneux *et al.*, 2014; Fauchier T, Hasseine L, Gari-Toussaint M, Casanova V, Marty PM, 2016) . An alternative approach is to target free nucleic acid within BAL specimens, by extracting direct from the BAL supernatant (Samuel *et al.*, 2011; Moodley, Tempia and Frean, 2017). The performance of PCP PCR can therefore be heavily influenced by the fraction of sample used, but also by the choice of DNA extraction methods used to recover nucleic acid from the clinical specimen.

Efforts to standardise PCR protocols are required to enable the comparison of performance across centres using PCR for diagnosis of PCP (Alanio *et al.*, 2012). One way to standardise performance is through the use of commercially available workflows for the isolation of nucleic acid (Springer *et al.*, 2013). Most commercially available extraction platforms utilise solid phase nucleic acid capture base technologies in the extraction protocols, see table 8.1. Solid phase capture of nucleic acid is efficient as it facilitates physical separation of nucleic acids from other biological components of the sample (Archer *et al.*, 2006). The impact of biological inhibitors such as complex polysaccharides and mucin are reduced, as they are not carried through the extraction process. The most common solid phase mechanism in automated DNA extraction systems is magnetic bead based capture, table 8.1.

**Table 8.1 Commercially available extraction platforms utilising magnetic bead based capture**

| **Platform** | **Manufacturer** |
|---|---|
| QiaSymphony | Qiagen |
| Bullet Pro | Diasorin |
| Maxwell | Promega |
| Liaison (Arrow) | Diasorin |
| EasyMag | Biomerieux |
| Kingfisher/Magmix | Thermofisher |

Magnetic bead-based capture of nucleic acid was first described in 1988 by Hultman and colleagues as a solid phase capture mechanism from which sequencing could be performed (Hultman *et al.*, 1989). A review of this technique was published a year later in Nature by Uhlen, who highlighted its potential application in DNA extraction from bacterial colonies of plasmid purification (Uhlen, 1989). Magnetic beads capable of binding DNA are manufactured in a variety of ways, but usually magnetically susceptible particles (e.g. iron oxide) are coated with synthetic or biological polymers, such as cellulose, polystyrene of silica (Berensmeier, 2006). Figure 8.1 demonstrates the basic principle behind magnetic bead based extraction. In summary, cell lysis is performed, releasing DNA into the supernatant. Streptavidin coated magnetic beads are added to the buffered solution and DNA is captured and immobilised on the beads. Using a magnet, beads are then separated from the solution, the supernatant is aspirated, and the beads are washed. DNA is then released from the beads through the addition of water or a buffered solution (e.g. tris-(hydroxymethyl)-aminomethane).

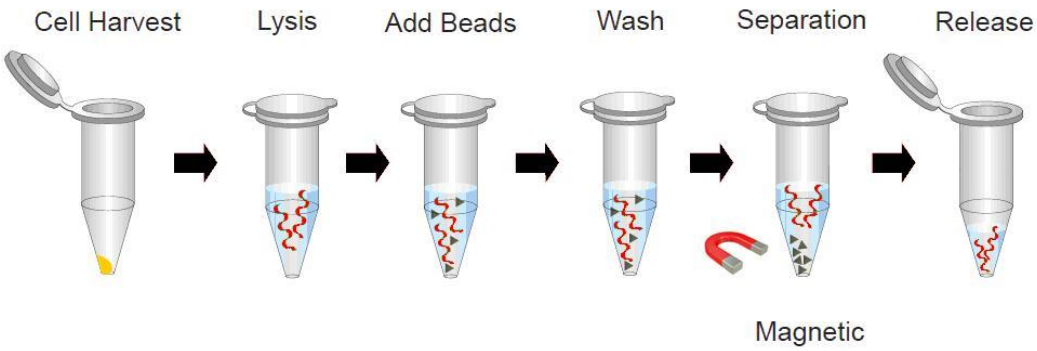



Figure 8.1 schematic of magnetic bead based DNA extraction (www.fairbiotech.com, 2017)

The Liaison IXT system (Diasorin) is an automated platform suited to low to medium throughput testing such as PCP PCR. The system can extract from 1 to 12 samples per run using pre-manufactured reagent cartridges, minimising reagent wastage and standardising the extraction process. The total run time for extraction is 45 minutes (manufacturers pre-set program). This extraction platform when coupled with a commercially available PCR, such as the MycAssay *Pneumocystis*™, provides a turnaround time to result in less than 3 hours. In comparison, a second automated system, BD MAX (Becton Dickinson, Fanklin Lakes, New Jersey), advances on the respective workflow by utilising a semi-closed system approach with nucleic acid extraction which takes place using individual reagent cartridges and is coupled with on-board PCR set up and processing. The benefits of using a system such as the BD MAX are mainly through savings in hands on time for the operator. In 2013 Dalpke and

colleagues developed and evaluated a *P. jirovecii* PCR for use on the BD MAX system (Dalpke *et al* , 2013). The first aim of this study was to investigate and compare the IXT/MycAssay and the BD MAX PCP workflows for the detection of *P. jirovecii* from BAL specimens.

## 8.2 Methods

### 8.2.1 Prospective clinical samples and plasmid control material;

BAL samples submitted to the Royal Free Hospital microbiology laboratory for the investigations of invasive fungal disease were included. 3mL was set as the minimum volume required for testing. Samples were stored at 4°C for 72 hours then centrifuged and two 1mL aliquots were frozen at -20°C prior to extraction. Plasmid control material containing the *P. jirovecii* large mitochondrial sub-unit (Mit LSU) specific for the MycAssay was sourced from Myconostica at a concentration of $1 \times 10^8$ copies/mL. For the BD MAX assay a positive-control plasmid containing a *P. jirovecii* HuMSG14 major surface glycoprotein gene (GenBank accession number AF033209) cloned into pCR2.1 was obtained from the research group of Dalpke *et al* (2013) at a concentration of $8 \times 10^8$ copies/mL. All plasmid was stored at -80°C prior to use in extraction studies.

### 8.2.2 Control samples from patients diagnosed with PCP

To analyse the sensitivity of the MycAssay *Pneumocystis*, DNA extracts and paired BAL aliquots were supplied by a collaborating centre led by Professor Oliver Schildgen at the Klinikum der Privaten Universität Witten-Herdecke mit Sitz in Köln, Institut für Pathologie, Cologne, Germany. DNA was extracted from 200µL BAL supernatant using the Maxwell RNA cartridge from Promega at the Klinikum der Privaten Universität Witten-Herdecke mit Sitz in Köln, Institut für Pathologie, Cologne, Germany. Both the extract and the native BAL were shipped on dry ice to the Royal Free microbiology laboratory and stored at -80°C until testing was performed.

### 8.2.3 Preparation of BAL samples obtained from the Royal Free Hospital laboratory

Samples were stored at 4°C for 72 hours then collected for testing. Viscous samples were pre-treated by mixing an equal volume of dithiothreitol (Sputasol, 1:1; Oxoid, Wesel, Germany) with the sample, followed by incubation at room temperature for 30

minutes. BAL samples were then centrifuged (4024g for 30 minutes) and the majority of supernatant discarded leaving 1mL to re suspend the pellet in. Samples were transferred to a screw cap 2mL tube (Sarstedt). If samples were not processed on the day of collection they were stored at -20°C until the day of testing (performed twice weekly). On the day of testing (following thawing if the samples were frozen), the samples were heat-treated on a dry heating block at 95°C for 30 minutes. After heat treating samples were split into two portions, 500µL was analysed using the Diasorin LIAISON® Ixt /MycAssay *Pneumocystis* assay and 500µL by the BD MAX system.

### 8.2.4 Diasorin LIAISON® Ixt nucleic acid extraction

Two types of extraction cartridges were used in this study for nucleic acid extraction. The first was a specific DNA extraction cartridge. This method of extraction targets whole cell DNA and is performed using the spun down pellet of BAL which was then re-suspended in lysis buffer specific to the cartridge kit. The second type of extraction cartridge targeted total nucleic acid and was designed to extract direct from the whole specimen, in this instance the neat BAL supernatant.

For prospective BAL samples obtained through from the Royal Free Hospital routine diagnostic laboratory, DNA extraction cartridges were utilised. 500µL of BAL concentrate was centrifuged at 8000g for 10 minutes. The supernatant was discarded and approximately 50µL of acid washed glass beads were added to the pellet. The pellet was disrupted through ribolysis on a FastPrep24 platform for 45 seconds. 240µL of lysis buffer 2 (Diasorin DNA extraction kit) was added immediately to the lysate along with 10µL of proteinase K. The tube was vortexed for 30 seconds then pulse centrifuged. Tubes were incubated for 10 minutes at 56°C on a dry hot block then transferred to the Diasorin Liaison IXT platform for extraction. Extraction was performed as per manufacturer's instructions. The program required 43 minutes from start to completion. Extracts were eluted into a 50µL volume.

For nucleic extraction from BAL obtained from the Institut für Pathologie, Cologne, Germany the total nucleic extraction cartridge was utilised. This method was in line with the extraction method used by the collaborating centre, a Promega Maxwell total nucleic acid cartridge. The selection of cartridge type was to allow a fair comparison across centres. Nucleic acid was extracted directly from the BAL supernatant without prior processing and eluted into a 50µL volume.

### 8.2.5 PCP PCR For *in vitro* Diagnostic Use MycAssay *Pneumocystis*

The MycAssay kit is separated into pouches, each pouch is sufficient to run 6 clinical samples, one positive and one negative control per run. Extraction was performed as per manufacturer's instructions

Reagent pouches were removed from the freezer and tubes 1 (master mix 1), 2 (master mix 2) and 3 (negative control) removed from their respective storage packaging. Tube 4 (positive control) was left in the storage pouch until required. The reagents were allowed to thaw for 5 minutes and briefly vortexed then pulse centrifuged. Smart Cycler reaction tubes (individual reactions) were placed into chilled support racks. To each tube 7.5µL of tube 1 and 7.5µL of tube 2 were added. To the final tube in sequence 10µL of the negative control was added and sealed. The reaction tubes were transferred to the extraction room along with the positive control. Each clinical sample extract was added to a single reaction tube for the Smart Cycler PCR. Once all clinical samples were added the positive control pouch was unsealed, the positive control was lightly vortexed and pulse centrifuged. 10µL of the positive control was added to the second to last tube in sequence and sealed. The reaction tubes were centrifuged for 10 seconds using a microfuge. A visual check was performed to ensure that no bubbles were present in the reaction mixtures. The PCR was performed according to the manufacturers cycling condition; 95°C for 10 minutes flowed by 39 cycles of 95°C for 15 seconds, 57°C for 50 seconds and 72°C for 20 seconds. A positive PCR was defined as a sigmoidal amplification curve with a cycle threshold value <35.

### 8.2.6 BD MAX Primers and Probes

The MSG primers amplify a 250-bp product from the target gene. The primer sequences (5'-3') for the *P. jirovecii* PCR were as follows gene MSG: MSG-fw, GAA TGC AAA TCC TTA CAG ACA ACA G, and MSG-rv, AAA TCA TGA ACG AAA TAA CCA TTG C. For detection, a dual labelled hydrolysis probe was used as follows: MSG probe, FAM-AGA CAT CGA CAC ACA CAA GCA CGT CT-BHQ1. Primer and probe stock solutions were prepared at 100µmol and stored at -20°C. The sample process internal control is included in the BD MAX ExK DNA-2 kit (BD Diagnostics) and carries the Drosophila melanogaster scaffold protein gene (GenBank accession number AC246497.1) cloned in a pUC119 vector sequence (GenBank accession number U07650).

### 8.2.7 Preparation of BD MAX PCR reagents

Primers and probes were purchased from Eurofins MWG Operon (Ebersberg, Germany). Primer and probe stock solutions were dissolved at 100µmol and stored at -20°C. The *P. jirovecii* target gene MSG primer sequences were as follows (5'- 3') MSG-fw, GAA TGC AAA TCC TTA CAG ACA ACA G, and MSG-rv, AAA TCA TGA ACG AAA TAA CCA TTG C (A H Dalpke, Hofko and Zimmermann, 2013). The dually labelled hydrolysis probe sequence was as follows: MSG probe, FAM-AGA CAT CGA CAC ACA CAA GCA CGT CT-BHQ1. The internal control probe was TET or Texas Red-CTA GCA GCA CGC CAT AGT GAC TGG C-BHQ2.

The probe/primer master mix was aliquoted at 12µL directly into conical snap-in tubes fitting the BD MAX extraction strip. A total of 200µL of the specimen was pipetted into the sample buffer tube (SBT) of the BD MAX ExK DNA-2 kit. The extraction was carried out following manufacturer's instructions. The PCP PCR assay was performed with the instrument in research mode using default settings for the extraction and the following PCR cycling protocol: 95°C for 60 seconds and 45 cycles of 98°C for 8 seconds and 58°C for 16.3 seconds. Fluorescence gains and thresholds were set for FAM (475/520nm). Quantitation was performed using a plasmid standard that was run once and saved as an external standard. For analysis in the PCR-only mode, 12µL of DNA or plasmid standard was added to the master mix and 12µL loaded manually into the PCR cartridge.

### 8.2.8 Analytical sensitivity

The analytical sensitivity of the workflows was determined by testing a tenfold dilution series of plasmid DNA of known concentrations from $10^6$-10 copies/µL in 10µL aliquots. The plasmid dilutions were analysed through each stage of the PCR workflows as follows:

1. Direct analysis through the PCR only. For the MycAssay *Pneumocystis* we were limited to processing three replicates due to the financial constraints of the study. For the BD MAX assay, as it was an in-house PCR, we were able to perform six replicates.
2. Spiked into and extracted from TE buffer and analysed by PCR.
3. Spiked into 1mL of BAL and extracted then analysed by PCR. For Diasorin Liaison IXT processing the pellet from the BAL was extracted using the DNA

cartridge and the remaining supernatant was extracted from using the total nucleic acid cartridge. For BD MAX processing the spiked BAL was inoculated directly into the extraction.

To measure reproducibility around the end point detection limit the $10^3$ copy/µL, $10^2$ copy/µL and 10 copies/µL were measured eight times by direct spiking into the PCR.

### 8.2.9 Linearity

To test the linearity of each PCR assay plasmid copies of $10^5$ to $10^2$ were analysed in triplicates through directly spiking the plasmid into the PCR reactions.

### 8.2.10 Inter-assay repeatability

To determine repeatability across different days of testing pooled *P. jirovecii* positive BAL in neat, 1:10 and 1:100 dilutions were processed three times on different days. The arithmetic mean (x), standard deviation (σ) and coefficient of variation (%CV) were calculated.

### 8.2.11 Evaluation of PCR performance when applied to characterised clinical samples

The performance of each PCR workflow was assessed though the analysis of samples from known PCP positive patients, obtained from an external laboratory at the Institute of Pathology, Hospital of the Private University Witten/Herdecke, Germany. The samples were anonymised at source and had been submitted for the investigation of fungal infection; redundant samples were retained under the umbrella of service development for which ethical consent was not required. The sample set contained 16 DNA extracts from BAL samples with 16-paired aliquots of supernatant from the same BAL samples. 10µL of the extracts were processed directly into PCR reactions for the BD MAX and the MycAssay *Pneumocystis* PCR. Due to sample volume restrictions the BAL supernatants had to be divided with samples 1-8 spiked into the BD MAX sample extraction buffer and processed through the BD MAX PCP PCR and samples 9-16 processed through the Diasorin Liaison IXT MycAssay *Pneumocystis* PCR workflow using the total nucleic acid extraction cartridges on the Diasorin LIAISON® Ixt.

As an additional measure, where enough volume was available, 13 of the extracts were also sent to an external site at NPHS Microbiology (Cardiff Heath Park, Wales) for analysis using a 23 rRNA assay specific for *P. jirovecii.*

### 8.2.12 Evaluation of PCR performance when applied to prospective clinical samples

Prospective analysis of BAL submitted to the Microbiology department was performed using the BD MAX and Diasorin LIAISON® Ixt/MycAssay *Pneumocystis* assays. Definitive PCP was diagnosed in the presence of a compatible clinical presentation of PCP, using the criteria below, and a positive Grocott's stain in BALF. To classify acute PCP lung disease the following criteria, adapted from those reported by Mühlethaler and colleagues (Mühlethaler *et al.*, 2012)

1. Proven PCP was made in those patient s with microscopy positive BAL (i.e., the presence of *P. jirovecii* trophic forms or cysts detected by MGG).
2. Possible PCP was diagnosed in deceased patients and/or patients having received an active drug against *P. jirovecii* for 5 days, if the following additional four elements were present: 1) compatibility of clinical signs (at least two symptoms: fever; dyspnoea; cough); 2) presence of hypoxia (arterial oxygen saturation, 93% and/or need for supplementary oxygen of 0.2L/min$^{-1}$ and/or mechanical ventilation); 3) compatible radiological findings (interstitial pattern and/or ground glass opacities on computed tomography); and 4) immunosuppression.
3. PCP was excluded in immunocompromised patients with acute lung disease compatible with PCP but have good outcome despite not receiving specific *P. jirovecii* therapy.

### 8.2.13 Statistical analysis

Linear regression was performed using Prism software, which calculated a correlation coefficient for the standard curve generated. The efficiency (*E*) of PCR is defined as the fraction of target molecules that are copied in one PCR cycle. Amplification efficiency (E) of the PCR was calculated from the slope of the standard curve obtained through processing plasmid dilutions using the following formula.

$$\mathbf{E = 10^{(-1/slope)}-1.}$$

It is possible to have efficiency >100% which most likely reflects the presence of PCR inhibitors or an excess in concentrated total nucleic acid within the sample.

## 8.3 Results

### 8.3.1 Analytical sensitivity and PCR efficiency

Analysis of known plasmid concentrations added directly to each PCR in replicates demonstrated 100% (3/3) reproducibility for the detection of the MitLSU target using the MycAssay *Pneumocystis* at 100 copies per reaction and 100% (6/6) reproducibility for the detection of the MSG target using BD MAX assay at 20 copies per reaction (table 8.2). Linear regression analysis, figures 8.2 and 8.3, of $C_T$ values obtained across the respective analyses demonstrated significant positive correlation coefficients for the MycAssay *Pneumocystis* (*r*=0.99) figure 8.2, and BD MAX PCP assays (*r*=0.99) figure 8.3. The median differences in CT values across tenfold dilutions of the target gene were 3.2 (range 3.5-2.8) for the MycAssay *Pneumocystis* and 2.8 (range 3.1-2.6) for the BD MAX PCP assay. PCR efficiency was calculated to be 97.2% for the MycAssay *Pneumocystis* and 100.5% for the BD MAX PCP PCR.

**Table 8.2 Mean $C_T$ values and *SD* for replicates (n=3) of plasmid dilutions spiked directly into MycAssay *Pneumocystis* PCR and the BD MAX *P. jirovecii* PCR**

| ***c*/mL** | **Reproducibility** | **ẋ** | **Σ** |
|---|---|---|---|
| **MycAssay *Pneumocystis*** | | | |
| 1E+8 | 100% (3/3) | 22.1 | 0.29 |
| 1E+7 | 100% (3/3) | 25.3 | 0.30 |
| 1E+6 | 100% (3/3) | 28.5 | 0.28 |
| 1E+5 | 100% (3/3) | 32 | 0.32 |
| 1E+4 | 100% (3/3) | 34.8 | 0.40 |
| 1E+3 | 66.6% (2/3) | 36.5 | 1.63 |
| **BD MAX *P. jirovecii* PCR** | | | |
| 5E+8 | 100% (6/6) | 17.2 | 0.33 |
| 5E+7 | 100% (6/6) | 20 | 0.26 |
| 5E+6 | 100% (6/6) | 23.1 | 0.27 |
| 5E+5 | 100% (6/6) | 26.1 | 0.48 |
| 5E+4 | 100% (6/6) | 28.4 | 0.57 |
| 5E+3 | 100% (6/6) | 31 | 0.75 |
| 5E+2 | ND | - | - |

When comparing $C_T$ values across the two PCR assays, measuring the same log concentration of plasmid, a significant difference was observed showing the MycAssay *Pneumocystis* has significantly lower $C_T$ values compared with the BD MAX PCR. The median $C_T$ value was 30.25 (IQR 24.5, 19.3) for the MycAssay and 24.6 (IQR 19.3, 29.1) for the BD MAX.

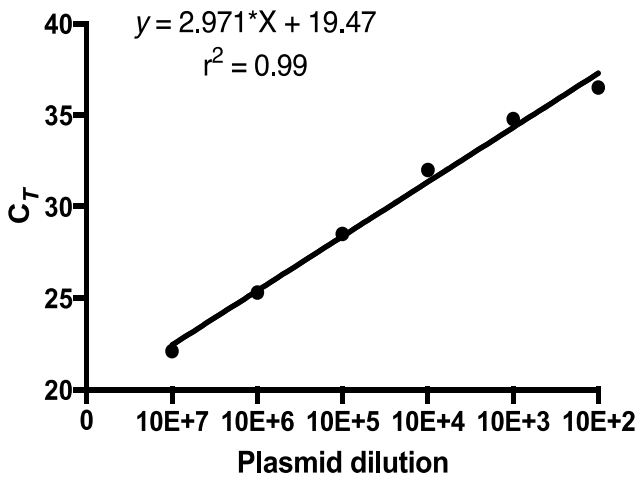



Figure 8.2 Linear regression of $C_T$ values for plasmid dilutions processed through the MycAssay *Pneumocystis* PCR.

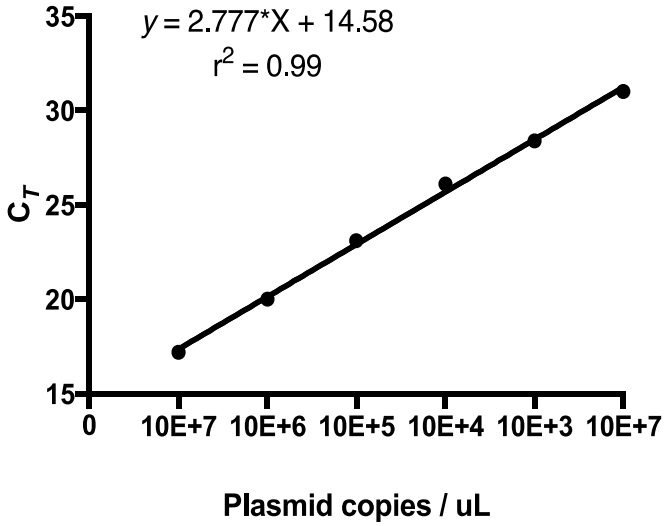



Figure 8.3 Linear regression of $C_T$ values for plasmid dilutions processed through the BD MAX *P. jirovecii* PCR.

### 8.3.2 Reproducibility at the limit of detection (LOD)

Replicate testing at the end point of detection demonstrated 100% reproducibility at the lowest input of 20 targets per reaction for the BD MAX PCP assay. The MycAssay *Pneumocystis* demonstrated 100% reproducibility at 1000 copies per reaction and 75% (6/8) reproducibility at 100 copies per reaction, table 8.3.

**Table 8.3 Analytical sensitivity of the BD MAX and MycAssay *Pneumocystis* PCR around the end point detection limit measured using plasmid control dilutions**

| BD MAX PCR | | | | | | MycAssay *Pneumocystis* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2E+3 | | 2E+2 | | 20 | | 1E+4 | | 1E+3 | | 1E+2 | |
| 100% | 8/8 | 100% | 8/8 | 100% | 8/8 | 100% | 8/8 | 100% | 8/8 | 87.5% | 6/8 |
| 26.1 | | 28.4 | | 31 | | 28.7 | | 31.5 | | 34.35 | |

### 8.3.3 Analytical sensitivity of the complete workflows for Diasorin Liaison IXT/MycAssay *Pneumocystis* and BD MAX PCP

Table 8.4 presents $C_T$ values obtained from plasmid dilutions extracted through each PCR workflow. When extracted on the Diasorin Liaison IXT plasmid target DNA was detected with 100% reproducibility using the MycAssay *Pneumocystis* PCR at an input of 100 copies. Target DNA was undetected at an input of 10 copies per sample. When plasmid target DNA was extracted on the BD MAX platform 100% reproducibility was observed with a total input of 2000 target copies, which decreased to 66.6% reproducibility (2/3) for a total input of 200 target copies.

**Table 8.4 Mean $C_T$ values and *SD* for replicates of plasmid dilutions processed through the BD MAX PCP workflow and the Diasorin Liaison IXT/MycAssay *Pneumocystis***

| Input | *Expected tC*/rxn | Reproducibility | ẋ C*q* | Σ |
|---|---|---|---|---|
| **MycAssay *Pneumocystis*** | | | | |
| 1E+6 | 2E+5 | 100% (3/3) | 25.6 | 0.62 |
| 1E+5 | 2E+4 | 100% (3/3) | 28.7 | 0.56 |
| 1E+4 | 2E+3 | 100% (3/3) | 31.27 | 0.25 |
| 1E+3 | 2E+2 | 100% (3/3) | 34.37 | 0.84 |
| 1E+2 | 20 | 100% (3/3) | 37.37 | 0.35 |
| 10 | 2 | 0% (0/3) | - | - |
| **BD MAX *P. jirovecii* PCR** | | | | |
| 2E+6 | 1.97E+5 | 100% (3/3) | 21.4 | 0.72 |
| 2E+5 | 1.97E+4 | 100% (3/3) | 23.8 | 0.25 |
| 2E+4 | 1.97E+3 | 100% (3/3) | 26.9 | 0.23 |
| 2E+3 | 1.97E+2 | 100% (3/3) | 30.2 | 0.29 |
| 200 | 19.7 | 66.7 (2/3) | 32.8 | 0.42 |

### 8.3.4 Analytical precision

Tables 8.5 and 8.6 detail $C_T$ *values and* calculated copies/mL for the respective conversion into cells/mL obtained from the analysis of a positive BAL serially diluted and tested on three different days through both workflows. %CV demonstrates that the two assays have a similar degree of variance across measurements from the same samples at the three dilutions tested. Quantification of *P. jirovecii* from the serially diluted BAL was linear in concordance with the tenfold dilutions prepared from the sample.

The measured copies per/ml was an average of 7.3 fold (6.3-8.42) higher through the IXT/MycAssay workflow compared with the BD MAX workflow. When normalized into

**Table 8.5 Mean $C_T$ values for dilutions of pooled *P. jirovecii* positive BAL processed through the BD MAX PCP and the Diasorin Liaison IXT/MycAssay *Pneumocystis* workflows on three different days**

| Dilution | Day 1 | Day 2 | Day 3 | ẋ | Σ |
|---|---|---|---|---|---|
| **MycAssay *Pneumocystis*** | | | | | |
| Neat | 20.6 | 21.2 | 20.9 | 20.5 | 0.96 |
| 1/10 | 23.1 | 24.3 | 24.1 | 23.8 | 0.64 |
| 1/100 | 26.3 | 27.2 | 27.5 | 27 | 0.62 |
| **BD MAX *P. jirovecii* PCR** | | | | | |
| Neat | 19.1 | 18 | 19.4 | 18.8 | 0.74 |
| 1/10 | 21.3 | 20.9 | 21.6 | 21.3 | 0.35 |
| 1/100 | 23.6 | 24.1 | 24.5 | 24.1 | 0.45 |

cells/mL both assays quantified the *P. jirovecii* burden in the dilutions at the same log10 quantities.

### 8.3.5 Clinical verification - analysis of samples from cases of PCP

In the first stage of the clinical verification process known positive samples donated by HPUW were analysed.

### 8.3.6 Analysis of DNA extracts

Concordance between the MycAssay *Pneumocystis* PCR and the qualitative results reported by HPUW from DNA extracts was 87.5% (14/16), table 8.7. The *q*PCR results, measuring copy numbers of target in the specimen, obtained by MycAssay *Pneumocystis* reported significantly lower number of target copy ($p<0.01$), on average $2.25x10^3$ fold target copies lower (range 6.6 – $1x10^4$ fold lower), compared with HPUW

*q*PCR results for DNA extracts, table 8.7 and figure 8.4. Two samples testing negative by MycAssay *Pneumocystis* PCR, were reported to contain $8.14x10^6$ and $8.9x10^6$ copies/mL by HPUW.

Concordance between the BD MAX PCP and the qualitative results reported by HPUW from DNA extracts was 31.3% (5/16). Significantly lower target copies ($p<0.01$) were detected using the BD MAX *P. jirovecii* PCR, on average $5.7x10^3$ fold lower (range 8 - $1.1x10^4$ fold), compared with HPUW qPCR results. *P. jirovecii* was not detected from 68.7% (11/16) of the extracts by the BD MAX PCP PCR despite the respective extracts measuring positive by HPUW ranging from $1.28x10^7$ to $7.32x10^{11}$ copies/mL as determined by the referring laboratory. When comparing qPCR results for the BD MAX IXT/MycAssay *Pneumocystis* workflow and converting into representative cells/mL both the BD MAX PCR and the MycAssay *Pneumocystis* demonstrated 80% (4/5) agreement for samples 1, 10 and 11 (table 8.7) when measuring the burden of *P. jirovecii* in extracts in the same Log10 concentrations. Measured cells/mL in sample 3 was discrepant measuring tenfold less by BD MAX PCR at $4.2x10^5$ compared with $1.31x10^6$-$1.3x10^7$ through the MycAssay *Pneumocystis* PCR, table 8.7).

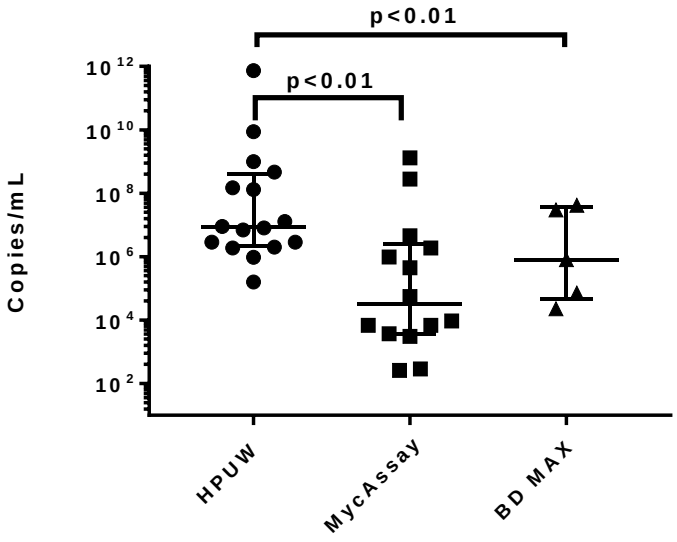



Figure 8.4 $C_T$ values obtained across the three methods processing DNA extracts from patients with proven PCP.

| Table 8.6 Quantitation analysis of copies per ml for dilutions of pooled *P. jirovecii* positive BAL processed through the BD MAX PCP and the Diasorin Liaison IXT/MycAssay *Pneumocystis* workflows on three different days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Dilution** | **Day 1** | **Day 2** | **Day 3** | **ẋ** | **Σ** | **CV** | **Cells/mL** | **ẋ fold change** |
| **MycAssay *Pneumocystis*** | | | | | | | | |
| Neat | 4.2E+8 | 2.6E+8 | 3.3E+8 | 3.34E+8 | 7.7E+7 | 0.23 | 7.7 E+6 | 1.4 |
| 1/10 | 6E+7 | 2.4E+7 | 2.8E+7 | 3.7E+7 | 1.9E+7 | 0.53 | 3.7 E+5 | 2.3 |
| 1/100 | 5.0E+6 | 2.5E+6 | 2.0E+6 | 3.2E+6 | 1.6E+6 | 0.51 | 3.2 E+4 | 2.3 |
| **BD MAX *P. jirovecii* PCR** | | | | | | | | |
| Neat | 6.6E+7 | 4.0E+7 | 5.2E+7 | 5.3E+7 | 1.3E+7 | 0.24 | 5.3E+5 | 1.5 |
| 1/10 | 8.5E+6 | 3.2E+6 | 3.7E+6 | 5.1E+6 | 2.9E+6 | 0.57 | 5.1E+4 | 2.5 |
| 1/100 | 6.1E+5 | 2.9E+5 | 2.3E+5 | 3.8E+5 | 2.1E+5 | 0.54 | 3.8E+3 | 2.4 |

Due to significantly different qPCR results observed between qPCR result from HPUW and the two PCRs in this study DNA extracts were referred to a third external laboratory, Bristol, for **23S** rRNA region (mtLSUrRNA) qualitative PCR, which is the same target as the MycAssay *Pneumocystis*. Figure 8.5 demonstrates $C_T$ values obtained with the MycAssay and the 23S assay. No significant difference (p0.69) was observed between the $C_T$ values obtained by these two assays, with a median $C_T$ of 34.5 (IQR: 27.2, 35.7) by 23S PCR and 33.3 (IQR: 27.2, 35.7) by MycAssay *Pneumocystis*.

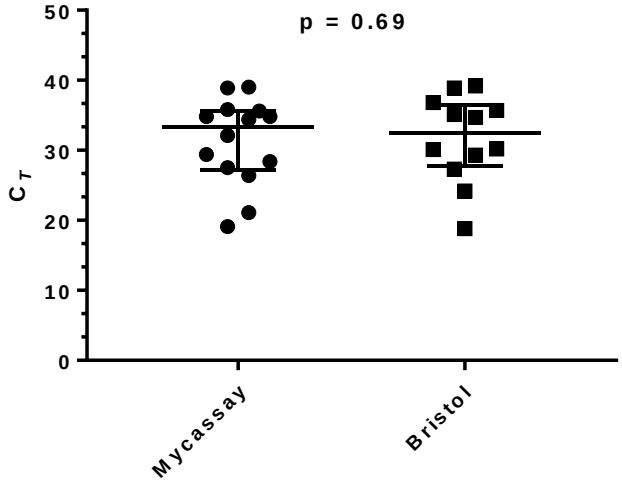



Figure 8.5 Comparison of $C_T$ values obtained from MycAssay and Bristol 23S PCR from DNA extracts.

### 8.3.7 BAL supernatants

When analysing BAL samples through the complete assay workflow including DNA extraction 87.5% (7/8) qualitative agreement was observed between the MycAssay *Pneumocystis* and HPUW, table 8.8. The IXT/MycAssay *Pneumocystis* workflow failed to detect *P. jirovecii* in a single BAL supernatant that had measured positive at $2.1x10^6$ copies/mL by HPUW. The remaining seven BAL supernatant samples all measured positive but the quantified target again measured significantly lower through the IXT/MycAssay workflow ($p<0.05$) with an average fold difference of $5.1x10^3$ (9.3x10-$1.7x10^4$ fold lower) compared with the referring laboratory results. When comparing qPCR results from extracted BAL supernatant with the original DNA extract results an average five-fold (range 1.2-9.4 fold) reduction in the *P. jirovecii* burden was measured, table 8.8.

The BD MAX PCP demonstrated 50% (4/8) qualitative agreement with HPUW, table 8.8. The BD MAX workflow measured 50% (4/8) of the BAL supernatant samples as negative with quantified target ranging between $9.68x10^5$ - $1.5x10^8$ copies/mL by HPUW. The remaining 50% (4/4) measured as positive by the BD MAX workflow, but again the quantified target was significantly lower, average 3.33 $x10^3$ fold (range 1.35x10-$1.33x10^6$ fold) compared with the quantification results supplied by the referring laboratory, table 8.8. Only two BAL samples, 1 and 3, measured positive that had positive results from the DNA extracts processed via PCR.

**Table 8.7 Results for BAL DNA extracts from known *P. jirovecii* positive tested by Institute of Pathology, Hospital of the Private University Witten/Herdecke, Germany then processed through the MycAssay PCR, BD MAX PCP PCR and Bristol 23S PCR**

| | HPUW | | | MycAssay MitLSU | | | | | BD MAX | | | | Bristol 23S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | ***c/mL*** | **cells/ml** | | **$C_T$** | ***c/mL*** | **cells/mL** | | **Fold difference[$]** | **$C_T$** | ***c/mL*** | **Cells/mL** | **Fold difference[$]** | **$C_T$** |
| | 100-1000 copies | | | | | 100-1000 copies | | | | | Single copy | | |
| 1 | 7.32E+11 | 7.32E+08 | 7.32E+09 | 21.1 | 2.80E+08 | 2.80E+05 | 2.80E+06 | 2.61E+03 | 20.6 | 3.0E+07 | 3.0E+05 | 2.44E+04 | NT |
| 2 | 8.90E+06 | 8.90E+03 | 8.90E+04 | *ND* | - | - | - | - | *ND* | - | - | - | ND |
| 3 | 8.70E+09 | 8.70E+06 | 8.70E+07 | 19.1 | 1.31E+09 | 1.31E+06 | 1.31E+07 | 6.6 | 20.3 | 4.20E+07 | 4.2E+05 | 2.07E+02 | 18.8 |
| 4 | 1.60E+05 | 1.60E+02 | 1.60E+03 | 34.8 | 6.90E+03 | 6.90E+00 | 6.90E+01 | 23.2 | *ND* | - | - | - | 35.1 |
| 5 | 2.00E+06 | 2.00E+03 | 2.00E+04 | 34.8 | 6.90E+03 | 6.90E+00 | 6.90E+01 | 2.90E+02 | *ND* | - | - | - | 35.7 |
| 6 | 9.68E+05 | 9.68E+02 | 9.68E+03 | 34.4 | 9.40E+03 | 9.40E+00 | 9.40E+01 | 1.03E+02 | *ND* | - | - | - | 36.8 |
| 7 | 1.50E+08 | 1.50E+05 | 1.50E+06 | 32.1 | 5.60E+04 | 5.60E+01 | 5.60E+02 | 2.68E+03 | *ND* | - | - | - | 30.1 |
| 8 | 2.90E+06 | 2.90E+03 | 2.90E+04 | 38.9 | 2.90E+02 | 2.90E-01 | 2.90E+00 | 1.00E+04 | *ND* | - | - | - | 39.2 |
| 9 | 1.30E+08 | 1.30E+05 | 1.30E+06 | 27.5 | 1.90E+06 | 1.90E+03 | 1.90E+04 | 68.4 | *ND* | - | - | - | 24.2 |
| 10 | 1.28E+07 | 1.28E+04 | 1.28E+05 | 26.4 | 4.60E+06 | 4.60E+03 | 4.60E+04 | 2.7 | 25.1 | 8.1E+05 | 8.1E+03 | 15.8 | 27.3 |
| 11 | 4.70E+08 | 4.70E+05 | 4.70E+06 | 29.4 | 4.50E+05 | 4.50E+02 | 4.50E+03 | 1.04E+03 | 29.4 | 2.3E+04 | 2.3E+2 | 2.04E+04 | 30.2 |
| 12 | 2.90E+06 | 2.90E+03 | 2.90E+04 | 39 | 2.60E+02 | 2.60E-01 | 2.60E+00 | 1.12E+04 | *ND* | - | - | - | 38.9 |
| 13 | 8.14E+06 | 8.14E+03 | 8.14E+04 | *ND* | - | - | - | - | *ND* | - | - | - | ND |
| 14 | 1.00E+09 | 1.00E+06 | 1.00E+07 | 28.4 | 9.80E+05 | 9.80E+02 | 9.80E+03 | 1.02E+03 | 28.1 | 7.1E+04 | 7.1E+2 | 1.41E+04 | 29.3 |
| 15 | 1.90E+06 | 1.90E+03 | 1.90E+04 | 35.8 | 3.10E+03 | 3.10E+00 | 3.10E+01 | 6.13E+02 | *ND* | - | - | | NT |
| 16 | 7.00E+06 | 7.00E+03 | 7.00E+04 | 35.6 | 3.70E+03 | 3.70E+00 | 3.70E+01 | 1.89E+03 | *ND* | - | - | | 34.7 |
| | | | | **87.5% (14/16)** | | | | | **87.5% (7/8)** | | | | **85.7% (12/14)** |

**Table 8.8 Results for BAL from known *P. jirovecii* positive tested by Institute of Pathology, Hospital of the Private University Witten/Herdecke, Germany then processed through the IXT/MycAssay and BD MAX PCP PCR complete workflow including extraction**

| | HPUW | | | MycAssay MitLSU | | | | | BD MAX | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Patient** | ***c/mL*** | **cells/mL** | | **$C_T$** | ***c/mL*** | **cells/mL** | | **Fold difference[$]** | **$C_T$** | ***c/mL*** | **Cells/mL** | **Fold difference[$]** |
| 1 | 7.32E+11 | 7.32E+08 | 7.32E+09 | NT | NT | - | - | | 26.4 | 3.2E+05 | 3.2E+03 | 2.29E+05 |
| 2 | 8.90E+06 | 8.90E+03 | 8.90E+04 | NT | NT | - | - | | 26.2 | 3.1E+05 | 3.1E+03 | 2.87E+00 |
| 3 | 8.70E+09 | 8.70E+06 | 8.70E+07 | NT | NT | - | - | | 22 | 1.00E+07 | 1.0E+05 | 8.70E+01 |
| 4 | 1.60E+05 | 1.60E+02 | 1.60E+03 | NT | NT | - | - | | 32.4 | 2.00E+03 | 2.0E+01 | 8.00E+00 |
| 5 | 2.00E+06 | 2.00E+03 | 2.00E+04 | NT | NT | - | - | | *ND* | - | - | |
| 6 | 9.68E+05 | 9.68E+02 | 9.68E+03 | NT | NT | - | - | | *ND* | - | - | |
| 7 | 1.50E+08 | 1.50E+05 | 1.50E+06 | NT | NT | - | - | | *ND* | - | - | |
| 8 | 2.90E+06 | 2.90E+03 | 2.90E+04 | NT | NT | - | - | | *ND* | - | - | |
| 9 | 1.30E+08 | 1.30E+05 | 1.30E+06 | 27.9 | 1.40E+06 | 1.40E+03 | 1.40E+04 | 9.29E+01 | NT | NT | - | |
| 10 | 1.28E+07 | 1.28E+04 | 1.28E+05 | 29 | 6.10E+05 | 6.10E+02 | 6.10E+03 | 2.10E+01 | NT | NT | - | |
| 11 | 4.70E+08 | 4.70E+05 | 4.70E+06 | 31.1 | 1.20E+05 | 1.20E+02 | 1.20E+03 | 3.92E+03 | NT | NT | - | |
| 12 | 2.90E+06 | 2.90E+03 | 2.90E+04 | 0 | - | - | - | | NT | NT | - | |
| 13 | 8.14E+06 | 8.14E+03 | 8.14E+04 | 38.2 | 4.90E+02 | 4.90E-01 | 4.90E+00 | 1.66E+04 | NT | NT | - | |
| 14 | 1.00E+09 | 1.00E+06 | 1.00E+07 | 30.9 | 1.40E+05 | 1.40E+02 | 1.40E+03 | 7.14E+03 | NT | NT | - | |
| 15 | 1.90E+06 | 1.90E+03 | 1.90E+04 | 38.7 | 3.30E+02 | 3.30E-01 | 3.30E+00 | 5.76E+03 | NT | NT | - | |
| 16 | 7.00E+06 | 7.00E+03 | 7.00E+04 | 35.8 | 3.20E+03 | 3.20E+00 | 3.20E+01 | 2.19E+03 | NT | NT | - | |
| **Total** | | | | **87.5% (7/8)** | | | | | **50% (4/8)** | | | |

### 8.3.8 Prospective clinical verification of PCP PCR

#### 8.3.8.1 Patient demographics

Table 8.9 and 8.10 detail the patient demographic characteristics for the overall patient population. When comparing gender, the ratio of males to females was higher in the PCP group. Not surprisingly, oxygen saturation was lower in the PCP group compared with the control group. Of note, oxygen saturation data was only available for seven patients in the control arm. Only two patients received anti-PCP therapy in the control group and only one patient had radiological findings consistent with PCP in the control arm but did not receive anti-PCP therapy during the episode around BAL sampling.

**Table 8.9. Patient demographics for BAL samples taken from 54 patients tested prospectively through two automated *P. jirovecii* PCR workflows; IXT/MycAssay *Pneumocystis* and BD MAX PCP PCR**

| | **Cases** | | **Controls** | |
|---|---|---|---|---|
| **Demographic** | **%** | ***n*** | **%** | ***n*** |
| % Male | 80 | 4 | 63.2 | 31 |
| Median age | 50 | | 56 | |
| Median $O_2$% | 88.5 | | 95.3 | |
| % Radiological evidence | 100 | 5 | 2 | 1 |
| % Anti-PCP therapeutic | 100 | 5 | 4 | 2 |

**Table 8.10 Underlying immunosuppressive conditions for 54 patients tested prospectively through two automated *P. jirovecii* PCR workflows; IXT/MycAssay *Pneumocystis* and BD MAX PCP PCR**

| **Underlying immunosuppressive condition** | **%** | ***n*** |
|---|---|---|
| Haemato-oncology | 20.4 | 11 |
| HIV (human immune deficiency virus) | 16.7 | 9 |
| GVHD (graft versus host disease) | 5.6 | 3 |
| Renal Transplant | 9.3 | 5 |
| Tuberculosis | 12.9 | 7 |
| High dose steroids | 5.6 | 3 |
| Liver Transplant | 3.7 | 2 |
| Other* | 25.9 | 14 |

#### 8.3.8.2 Clinical samples

In total, 57 BAL samples from 54 patients were analysed prospectively by both *P. jirovecii* PCR workflows. Three samples were submitted from a second BAL taken from three of the patients more than two weeks apart, so were included as independent samples.

When tested by the IXT/MycAssay workflow 10.5% (6/57) samples were positive, 80.7% (46/57) of samples were negative and 8.8% of samples were inhibited (5/57). When tested by the BD MAX workflow 7% (4/57) of samples were positive, 84.2% (48.57) of samples were negative and 5.3% (3/57) of samples were inhibited.

#### 8.3.8.3 Classification of patients: Proven, Probable and control

According to the clinical criteria, detailed in section 8.2.12, for defining PCP infection 3.7% (2/54) of the patients were classified as probable PCP infection, 5.6% (3/54) as proven infection table 8.11. The remaining 90.7% (49/54) patients were classified as having no evidence of PCP infection.

**Proven infection:** Two patients were defined as having proven infection through the positivity of Grocott's staining on BAL samples positive for *Pneumocystis*. Patient 1 was an AML patient with radiology suggestive of PCP, who received anti-PCP therapy and had an oxygen saturation of 92% at the time of presentation, table 8.11. The measured load of *P. jirovecii* in the BAL processed for PCR was 1.5-15 cells per mL by IXT/MycAssay but was not detected by the BD MAX workflow.

Patient 2 was an HIV patient presenting with hypoxia, with ground glass changes on CT, and who had received anti-PCP therapy. The measured *P. jirovecii* burden in BAL was 3.5 E+05-E+06 by IXT/MycAssay and 7.1 E+05 by the BD MAX workflow table 8.11.

**Probable infection**: All patients with probable PCP received cotrimoxazole treatment around the time of the BAL sampling and all had ground glass opacification consistent with PCP infection noted on their radiological reports. All had hypoxia at the time of presentation noted in clinical records; oxygen saturation readings were available for 2 of the three patients as detailed in table 8.11.

- Patient 3, a renal transplant patient, had a measured burden of 2.3 E+03 – 2.3 E+04 *P. jirovecii* cells/mL by IXT/MycAssay *Pneumocystis* and 1.1 E+05 by BD MAX.
- Patient 4, an HIV patient, had a measured burden of 1.3E+04 – 1.3 E+05 P. jirovecii cells/mL by IXT/MycAssay *Pneumocystis* and 6.2 E+02 by BD MAX.
- Patient 5, receiving high dose corticosteroids for an underlying condition, had a measured burden between 28 - 280 *P. jirovecii* cells/mL by IXT/MycAssay *Pneumocystis* but no cells were detected by BD MAX.

**No evidence of PCP infection**; The final positive PCR result was measured in patient 6 who presented with atypical changes on radiology and was not considered to be at risk of PCP. Underlying immunosuppression was the administration of high dose corticosteroids. No anti-PCP therapy was administered to this patient and *P. jirovecii* microscopy was not requested. The measured burden of *P. jirovecii* cells/mL by IXT/MycAssay *Pneumocystis* was 8.2 E+04-8.2 E+05 and was 3.4 E+05 by BD MAX, table 8.11.

**Table 8.11 Summary of clinical criteria and PCP definitions for six patients with positive *P. jirovecii* PCR results from BAL**

| Patient | Sex | Classification | Age | $O_2$% | Radiology | Anti-PCP therapy | Immune-suppression | Grocott's | IXT/MycAssay | BD MAX |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | Proven | 56 | 91 | GGO | Cotrimoxazole Pentamidine | Acute Myeloid Leukemia | Positive | 1.5-15 | ND |
| 2 | M | Proven | 30 | 92 | GGO | Cotrimoxazole | HIV infection | Positive | 3.5 E+05-3.5E+06 | 7.1 E+05 |
| 3 | M | Probable | 54 | 91 | GGO | Cotrimoxazole | Renal Transplant | Negative | 2.3E+03-2.3E+04 | 6.2 E+02 |
| 4 | M | Probable | 56 | 80 | GGO | Cotrimoxazole | HIV infection | Negative | 1.3 E+04-1.3 E+05 | 1.1 E+04 |
| 5 | F | Probable | 58 | ND* | GGO | Cotrimoxazole | High dose corticosteroids | Negative | 28-280 | ND |
| 6 | M | Control | 72 | ND | Atypical changes | No | High dose corticosteroids | ND | 8.2 E+04 -2 E+05 | 3.4 E+05 |

GGO= ground glass opacification, $O_2$ = oxygen saturation

#### 8.3.8.4 Performance of PCP PCR compared with clinical diagnosis as the gold standard

The IXT/MycAssay *Pneumocystis* workflow demonstrated a sensitivity and NPV of 100% (5/5, 95% CI: 56.6, 100) and 100% (46/46, 95% CI: 92.3, 100) respectively. Specificity was 97.9% (46/47, 95% CI: 88.9, 99.6) and PPV was 83.3% (5/6, 95% CI: 43.7, 97). Diagnostic accuracy was 98.1% (51/52, 95% CI: 89.9, 99.7) with clinical diagnosis as the gold standard; a single false positive result was observed detailed in table 8.12. This patient was on high dose corticosteroids and had atypical pneumonia on radiology but was not suspected to have PCP, oxygen saturation was unavailable and the patient did not receive anti-PCP therapy.

The BD MAX PCP PCR workflow demonstrated a sensitivity of 60% (3/5, 95% CI: 23.1, 88.2) and PPV of 75% (3/4, 95% CI: 30, 95.4) and a specificity and negative predictive value of 98% (48/49, 95% CI: 89.3, 99.6) and 96% (48/50, 86.5, 98.9) respectively. 94.4% (51/54, 95% CI: 84.9, 98.1) agreement was achieved with clinical diagnosis as the gold standard. The same false positive was detected as with the IXT/MycAssay *Pneumocystis* workflow. Two false negative results were recorded, one in a proven case of PCP and one in a probable case of PCP, table 8.12

#### 8.3.8.5 Performance of PCP PCR compared with microscopy as the gold standard

The IXT/MycAssay *Pneumocystis* workflow demonstrated a sensitivity and NPV of 100% (2/2, 95% CI: 32.2, 100) and 100% (46/46, 95% CI: 92.3, 100) respectively when compared with microscopy. Specificity was 92% (46/50, 81.2, 996.9) and PPV was 33.3% (2/6, 95% CI: 9.7, 70). Less agreement was achieved with microscopy compared to clinical diagnosis 92.3% (48/52, 95% CI: 81.3, 97) Table 8.11. Four false positives were observed in three patients with probable infection and one control patient detailed in table 8.12.

The BD MAX PCP PCR workflow demonstrated a sensitivity of 50% (1/2, 95% CI: 9.5, 90.6) and PPV of 25% (1/4, 95% CI: 4.6, 70) and specificity and negative predictive value of 94.2% (49/52, 84.4, 98) and 98% (49/50, 95% CI: 89.5, 99.7) respectively, table 8.11. With clinical diagnosis as the gold standard 92.6% (50/54, 95% CI: 82.5, 97.1) agreement was achieved. Three false positive results were observed, all in probable cases of PCP. One false negative result occurred in a microscopy positive

proven case of PCP, from which a high $C_T$ of 33 was measured by the IXT/MycAssay *Pneumocystis* workflow.

**Table 8.12 Performance characteristics of the IXT/MycAssay *Pneumocystis* and BD MAX PCP PCR from clinical specimens when measured against clinically defined proven/probable PCP infection and microscopy as gold standards**

| | IXT/MycAssay | | | BD MAX PCP | |
|---|---|---|---|---|---|
| | Clinical diagnosis | | | | |
| | **Positive** | **Negative** | | **Positive** | **Negative** |
| **Positive** | 5 | 1 | | 3 | 1 |
| **Negative** | 0 | 46 | | 2 | 48 |
| | | | | | |
| **Accuracy** | 98.1% | 89.9, 99.7 | | 94.4% | 84.9, 98.1 |
| **Sensitivity** | 100% | 56.6, 100 | | 60% | 23.1, 88.2 |
| **Specificity** | 97.9% | 88.9, 99.6 | | 98 | 89.3 99.6 |
| **PPV** | 83.3% | 43.7, 97 | | 75 | 30, 95.4 |
| **NPV** | 100% | 92.3, 100 | | 96 | 86,5, 98.9 |
| | Microscopy | | | | |
| | **Positive** | **Negative** | | **Positive** | **Negative** |
| **Positive** | 2 | 4 | | 1 | 3 |
| **Negative** | 0 | 46 | | 1 | 49 |
| | | | | | |
| **Accuracy** | 92 | 81.8 97 | | 92.6 | 82.5, 97.1 |
| **Sensitivity** | 100 | 32.2, 100 | | 50 | 9.5, 90.6 |
| **Specificity** | 92 | 81.2, 96.9 | | 94.2 | 84.4, 98 |
| **PPV** | 33.3 | 9.7, 70 | | 25 | 4.6, 70 |
| **NPV** | 100 | 92.3, 100 | | 98 | 89.5, 99.7 |

## 8.4 Discussion

The aim of this chapter was to evaluate automated DNA extraction alongside PCR for the diagnosis of PCP. Two workflows were investigated. The first was a stand-alone automated DNA extraction platform, the IXT, alongside a commercially available MycAssay *Pneumocystis* PCR. The second workflow was a closed system automated platform, BD MAX, performing both DNA extraction and PCR on board in a continuous workflow. This study has demonstrated that both IXT/MycAssay *Pneumocystis* had a very high specificity and NPV of 97.9 (955 CI: 88.9, 99.6) and 100 (95% CI: 56.6, 100) and the BD MAX PCR had a high specificity and NPV of 98% (95% CI: 89.3 99.6) and 96% (95% CI: 86.5, 98.9) for the exclusion of *Pneumocystis* pneumonia when

compared with clinical diagnosis as the gold standard. The sensitivity and PPV of the IXT/MycAssay workflow at 100% (95% CI: 34.2, 100) and 83.3% (95% CI: 43.7, 97) was superior to the BD MAX PCR in this study 60% (95% CI: 23.1, 88.2) and 75% (95% CI: 30, 95.4). Several studies utilising MycAssay *Pneumocystis* have supported these findings with high NPV ranging between 99-100% and sensitivities ranging between 93-100% (Hauser *et al.*, 2011; McTaggart, Wengenack and Richardson, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b). However, the reported specificity and PPV across these studies varies considerably ranging between 79.1-100% and 33.3-80% respectively (Hauser *et al.*, 2011; McTaggart, Wengenack and Richardson, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b). There are several contributing factors affecting specificity and PPV when using PCR to diagnose PCP.

### 8.4.1 Verification of the analytical performance of *P. jirovecii* PCR

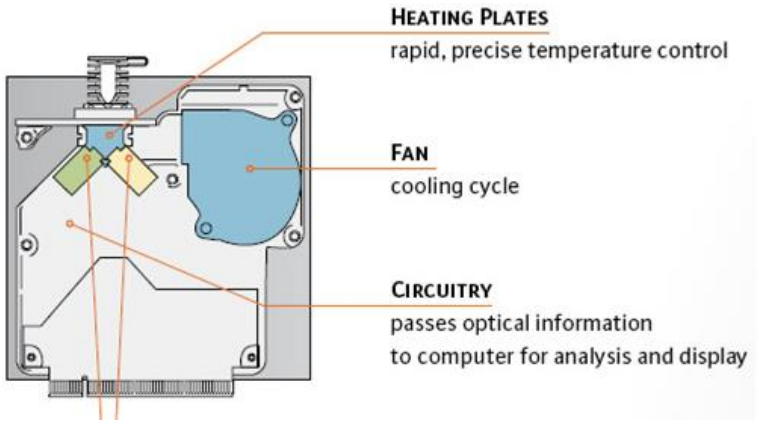
In this study the linearity, sensitivity and the precision (inter and intra assay reproducibility) of both PCR workflows were investigated. A high degree of linearity was observed across serial dilution of plasmids processed through both assays indicating accuracy at the upper and lower detection limits of both assays. The calculated PCR efficiencies for both PCR's were not significantly different, 100% vs 97.2% for the Myconostica vs the BD MAX respectively. Furthermore both assays demonstrated excellent intra and inter-assay reproducibility however, differences in performance were observed when assessing the analytical LOD of each PCR the MycAssay.

The MycAssay *Pneumocystis* PCR was 10-100 fold less sensitive than the BD MAX PCP PCR which was reproducibly positive at 20 plasmid copies input, which is similar to previous published sensitivities using this assay (Alexander H. Dalpke, Hofko and Zimmermann, 2013). In table 8.2 average $C_T$ values obtained from the two PCRs demonstrate that the BD MAX has earlier $C_T$ values compared with the Myconostica assay, at $10^6$ copies $C_T$ values were 25.3 for the MycAssay v's 20for the BD MAX. At the $10^3$ concentrations the MycAssay $C_T$ was 37 reaching the end point of the assay. A ten-fold lower plasmid concentration would have had a $C_T$ value exceeding the set cycling conditions of 40 cycles and was therefore undetected. The differences in PCR performance may be a result of the mechanical hardware or the molecular design of PCR primers and probes (Reynisson *et al.*, 2006; Buh Gašparič *et al.*, 2010; Navarro *et al.*, 2015).

The detection system within the hardware of the BD MAX and the Smartcycler used for MycAssay are proprietary so comparisons cannot be made but there are some obvious differences between the two systems that may contribute to variance in performance. The BD MAX system utilizes a microfluidic chamber that performs a PCR from a reaction volume of 4µL, (diagram 8.6.b). The Smart cycler PCR tubes contain the reaction in a thin window; at 5-6 times the surface area of the BD MAX chamber therefore the reaction is spread over a wider surface. The thermal block on the smart cycler is also situated underneath the tube compartment rather than in direct contact with the reaction window of the tube as with the BD MAX, diagram 8.6.a. The differences in the design of the PCR systems could have contributed to the decreased sensitivity of the MycAssay when amplifying plasmid copies figure 8.6.a. The significance of the difference in PCR sensitivity is normalised through the choice of target amplified in each assay. The MycAssay targets the MitLSU, a multicopy target at 100-1000 copies per cell (as specified by the manufacturer). The BD MAX system detects the major surface glycoprotein (MSG) a single copy gene within each cell therefore increased sensitivity is required (Alexander H. Dalpke, Hofko and Zimmermann, 2013).

The rate of inhibition on the SmartCycler platform was higher, 8% (7/43) than the 4.7% seen using the BD MAX PCR. Chemistry of the Myconostica PCR master mix may have impacted on performance of the assays. The BD MAX utilizes a hydrolysis TaqMan whereas the Myconostica *P. jirovecii* utilizes a hairpin molecular beacon. A previous study have described comparable results using either probe design (Buh Gašparič *et al.*, 2010) providing optimization is performed especially in the instance of molecular beacons (Buh Gašparič *et al.*, 2010). A comparative study observed the lowest efficiency using the molecular beacon design compared with other probes and it was stated that this probe design was more sensitive to changes in the PCR reaction, such as inhibitors (Andersen *et al.*, 2006). Freeze thawing the extracts led to a reduction in inhibition (due to the known detrimental effect freeze thawing has on an residual protein/enzymatic material in the extracts) and could be added to the workflow ahead of performing the PCR, but would lead to an extension of the turnaround time, which is not desirable considering the extended extraction process of almost 4 hours

a)




b)


Diagram 8.6 a) design of the smart cycler unit demonstrating the position of the thermal heating block b) a BD MAX cartridge demonstrating the cylindrical reaction chambers that hold the 4µL reaction.

Plasmid dilutions processed from extraction to PCR allowed the impact of DNA extraction on sensitivity of the workflow to be assessed. In contrast to the findings of direct PCR analysis, the BD MAX workflow was ten-fold less sensitive than the IXT/MycAssay workflow, which includes the extraction component, with an LOD of approximately $10^3$ copies per mL compared to $10^2$ for the IXT/MycAssay workflow. Dalpke and colleagues observed the same sensitivity using the BD MAX PCR workflow with 100% detection at $10^3$ c/mL versus 33% detection at $10^2$ c/mL (Alexander H. Dalpke, Hofko and Zimmermann, 2013). The loss in analytical sensitivity when processing samples through the entire workflow using the BD MAX is most likely a result of the analytical steps built into the extraction process on the BD MAX. Figure 8.7 details the pipetting steps included in each PCR workflow, highlighted are the steps contributing to a dilution effect on the final target extracted from the sample. When extracted through the BD MAX system at best only 10% of the target DNA extracted from the sample will be transferred to the reaction, compared with 20% using the IXT/Myconostica workflow.

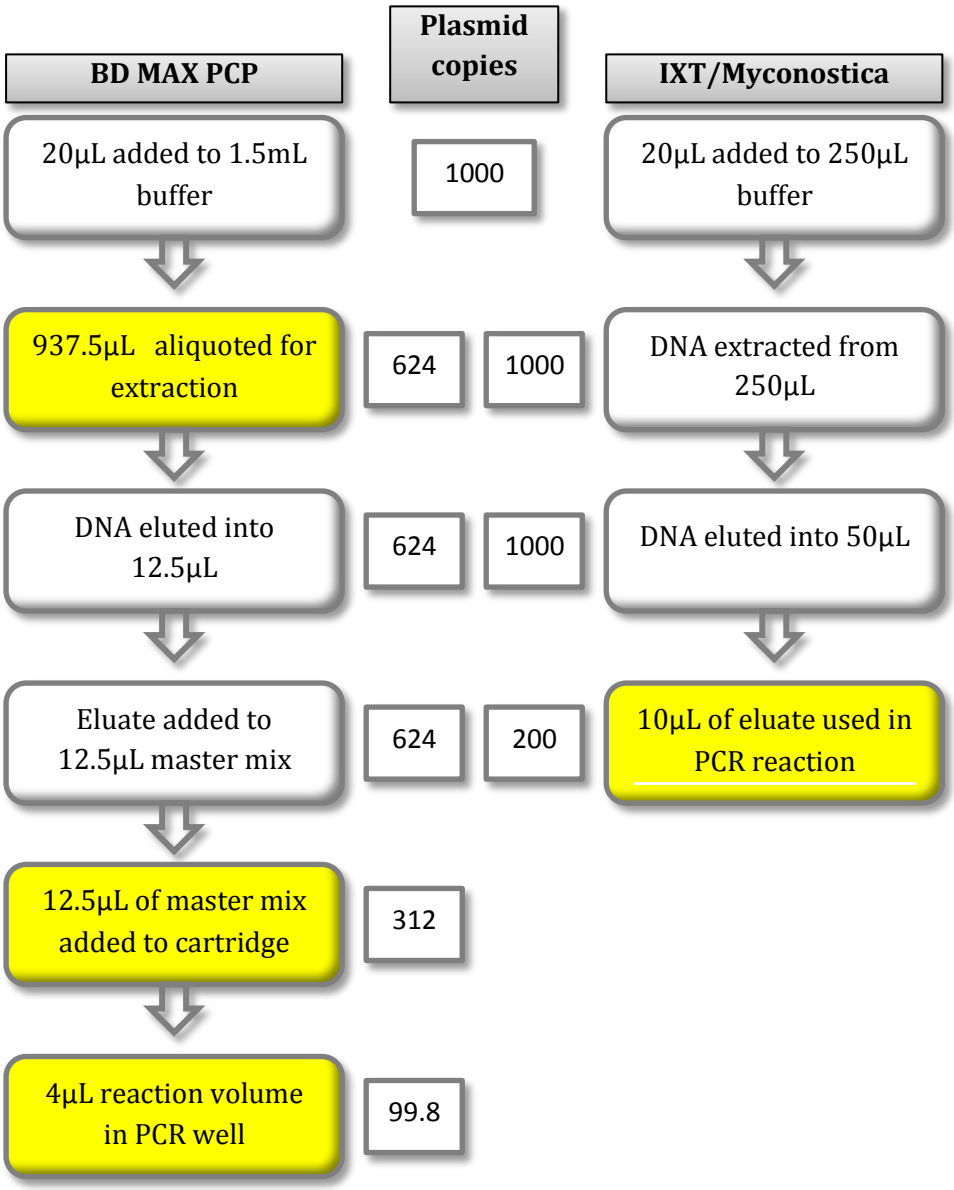



Figure 8.7. Pipetting workflow for the BD MAX PCP PCR and IXT/Myconostica.

This study clearly demonstrates that when evaluating the performance of PCR for diagnostic purposes it is important to evaluate each component of the workflow so that it is possible to understand the limitations of each stage of the process. Despite the BD MAX PCR being highly sensitive the loss of target through the extraction makes it the less sensitive workflow analytically. This is an important factor to consider when analysing clinical specimens, so that one can rationalize when difference in performance observed from clinical samples. Differences in PCR performance were observed when processing extracts from clinical samples and BAL supernatant from known positive patients. DNA extracts.

### 8.4.2 Clinical performance of PCP PCR

This study has demonstrated that both IXT/MycAssay *Pneumocystis* and the BD MAX PCR could be used with confidence for the exclusion of *Pneumocystis* pneumonia with high NPV and sensitivity. Several studies utilising MycAssay *Pneumocystis* have supported these findings with high NPV ranging between 99-100% and sensitivities ranging between 93-100% (Hauser *et al.*, 2011; McTaggart, Wengenack and Richardson, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b).

The sensitivity and PPV in this study was 100% (CI: 56.6, 100) and 83.3% (43.7, 97) for the MycAssay *Pneumocystis* and for the BD MAX PCP PCR was 60% (23.1, 88.2) and 75% (30, 95.4) when compared against clinical diagnosis of proven or probable disease as the gold standard. Our study was limited in that only two specimens from proven cases of PCP were included, and three cases of probable disease. Reported specificity and PPV across studies utilising PCP PCR varies considerably ranging between 79.1-100% and 33.3-80% respectively (Hauser *et al.*, 2011; McTaggart, Wengenack and Richardson, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b). There are several contributing factors affecting specificity and PPV when using PCR to diagnose PCP. The burden of *P. jirovecii* in colonisation is poorly defined and may be transient with human-to-human transmission occurring (Gigliotti and Wright, 2012). An autopsy study using a nested PCR from lung tissue samples reported 64.9% of adults had detectable *P. jirovecii*. For 68% of the samples positive by PCR, colonization with *P. jirovecii* was confirmed by IF microscopy but only with enhanced analysis of the IF slides (90 minutes per slide) (Ponce *et al.*, 2010). Distinguishing infection from colonization in the laboratory is challenging. Confidently diagnosing PCP infection based on laboratory tests alone relies on detecting a significant burden of *P. jirovecii* from clinical specimens. This is the reason why microscopy can be the preferred method of detection as IF staining has a LOD of $1.5 \times 10^3$ cfu/mL of organisms from BAL (Alanio, Desoubeaux, *et al.*, 2011) and Grocott's staining being less sensitive and having a detection limit of $10^4$ - $10^5$ cfu/mL (Baselski and Wunderink, 1994). When *P. jirovecii* is detected using light or fluorescence microscopy a high PPV for infection can be expected as a positive result indicates a significant burden of organism in the specimen and can be used to definitively diagnose PCP. It is reasonable to hypothesise that increased sensitivity using PCR for *P. jirovecii* detection leads to increased laboratory diagnosis of PCP compared with GMS microscopy.

In our study 40% (2/5) of BAL samples positive by PCR were positive by GMS microscopy. PCR results for one of the patients was a very low positive by MycAssay

with only 150-1500 copies/mL the equivalent of 1-10 cells/mL of BAL being measured, this sample was negative by BD MAX PCR. The second positive patient had a high positive PCR result at 3.5 x $10^6$ copies per µL equivalent to 3.5 x $10^3$-3.5 x $10^4$ cells per mL by MycAssay and 7 x $10^5$ copies by BD MAX PCR. The remaining three samples positive on PCR but negative by GMS microscopy had burdens of *P. jirovecii* ranging from 3 cells per mL to 1 x $10^3$ cells per mL as measured by MycAssay PCR, two of which were also positive at the same level by BD MAX PCR. From this limited data there doesn't appear to be a positive correlation with increased burden of *P. jirovecii* in a BAL specimen and detection by GMS stainin as the sample with the lowest PCR positive signal was detected by GMS staining. In larger studies PCR has been shown to be more sensitive than Grocott's staining and IF for the detection of *P. jirovecii* from clinical samples (Gupta *et al.*, 2008; Chumpitazi *et al.*, 2011; Hardak *et al.*, 2012; Robert-Gangneux *et al.*, 2014). This is particularly the case in the non-HIV patient populations (Hardak *et al.*, 2012; Robert-Gangneux *et al.*, 2014). In 2008 Gupta and colleagues demonstrated that a conventional single round PCR amplifying the Major Surface Glycoprotein (MSG) gene detected *P. jirovecii* in 11% of patients with a clinical suspicion of PCP compared with 4% using GMS (Gupta *et al.*, 2008). In the respective study *P. jirovecii* was not detected in the control group even after the application of a highly sensitive nested PCR (Gupta *et al.*, 2008). Similarly Chumpitazi *et al* demonstrated that using a quantitative PCR *P. jirovecii* could be detected in 95.8% of samples from patients with confirmed PCP compared with 66.7% using GMS (Chumpitazi *et al.*, 2011). More strikingly it has also been demonstrated that GMS only detected *P. jirovecii* in 20.7% of specimens compared with 96.2% using PCR from patients considered having probable PCP infection with a clinical presentation consistent with infection (Matsumura *et al.*, 2012). Furthermore, this study demonstrated resolution of pulmonary infiltrates after anti-PCP treatment in the PCR positive group (Matsumura *et al.*, 2012). Increased sensitivity using PCR for the detection of *P. jirovecii* also leads to increased laboratory diagnosis of PCP compared with conventional IF microscopy.

Muhlethaler *et al* detected three additional cases of probable PCP as defined by strict clinical criteria that tested negative by IF (Mühlethaler *et al.*, 2012). Orsi and colleagues demonstrated that the MycAssay *Pneumocystis* assay detected an additional case of PCP in a non-HIV patient negative by IF (Orsi *et al.*, 2012). Increased sensitivity using PCR for *P. jirovecii* detection also leads to increased laboratory diagnosis of PCP in the paediatric population. Samuel and colleagues reported a significant increase of 47.6% (154/323) in the detection of *P. jirovecii* from upper and lower respiratory tract

specimens in a cohort of HIV-infected African children hospitalized with a high clinical suspicion of PCP using PCR (noting only 6.6% of controls were positive in this study (Samuel *et al.*, 2011). Several other studies have also demonstrated increased detection of *P. jirovecii* from patients diagnosed clinically with PCP using PCR compared with microscopy (Hauser *et al.*, 2011; Hardak *et al.*, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b; Guigue *et al.*, 2014; Maillet *et al.*, 2014; Nakashima *et al.*, 2014) However, increased sensitivity can also lead to detection of colonization in patients with no attributable symptoms of PCP.

Across the five studies evaluating PCR the mean PPV was 76.7% (range 59%-95.5%) (Chumpitazi *et al.*, 2011; Hauser *et al.*, 2011; Matsumura *et al.*, 2012; Orsi *et al.*, 2012; Seah *et al.*, 2012b). In patients with proven PCP usually defined as being positive on microscopy in combination with clinical diagnosis the PPV reached 100% across most studies (Hauser *et al.*, 2011; Lu *et al.*, 2011; McTaggart, Wengenack and Richardson, 2012; Fan *et al.*, 2013; Matsumura *et al.*, 2014). Using PCR to distinguish between patients with probable PCP and colonisation can be more complicated. Using quantitative PCR Matsumara and colleagues demonstrated no significant difference in the measured copies/mL from the BAL of patients with probable PCP infection and those who were colonized (Matsumura *et al.*, 2012). Using the MycAssay *Pneumocystis* assay Hauser and colleagues observed positivity in seven patients without any clinical evidence of PCP (Hauser *et al.*, 2011). In the respective study $C_T$ values were significantly lower (stronger positive) for the probable PCP group than the colonized group (median: 29.4 v's 34.2). Seah and colleagues chose to define a true positive as one that could be confirmed with microscopy or a second *P. jirovecii* PCR when evaluating the MycAssay *Pneumocystis* assay (Seah *et al.*, 2012b). In the respective study the PPV was 70.5% with 17 positive results not confirmed by a second assay. The $C_T$ values of true positive results confirmed by a second PCR were significantly lower (31.79 +/- 2.87) than false positive results that could not be confirmed by a second test (36.3 +/- 1.81). Distinguishing between colonisation and infection based on a $C_T$ threshold cut of ≤25 for infection and 26-36 for colonisation was also proposed by Rudramurthy and colleagues, using the major surface glycoprotein as a target (Rudramurthy SM, *et al* 2017). Caution must be used when interpreting the strength of a positive result based on the $C_T$ values alone as several factors can influence the values obtained. $C_T$ values on one system can be significantly different to those on a differing system at the same log concentrations. This was demonstrated in our study. Qualitative PCR is therefore limited by an inability to accurately reflect the burden of organisms within specimens. Quantitative PCR

addresses this limitation, running standards alongside clinical samples it is possible to quantify the target copies detected and quantify the positivity of a sample.

The use of quantitative PCR for the characterisation of *P. jirovecii* infection has been reported in several studies (Lu *et al.*, 1995; Alanio, Desoubeaux, *et al.*, 2011; Chumpitazi *et al.*, 2011; Maillet *et al.*, 2014; Matsumura *et al.*, 2014). By quantifying *Pneumocystis* cells it may allow clinicians to make a decision regarding the significance of a positive result (Lu *et al.*, 1995). It presents an opportunity to not only define the burden of organisms within a sample but to also define thresholds for infection and colonization (Robert-Gangneux *et al.*, 2014). Across published studies using quantitative PCR there is a common approach in which a lower threshold is established to define colonization and a higher threshold is established to define infection, leaving a 'grey area' in which a positive result cannot definitively be used to classify a patients disease status and needs to be interpreted in the context of a patient's clinical presentation. Table 8.12 details five studies defining thresholds for *P. jirovecii q*PCR interpretation. In study 1 the MSG single copy gene was used and a threshold <3160 equivalent to 1E+04 cells per mL was used to define colonisation and >31600 copies equivalent to 1E+06 cells per mL used to define infection (Maillet *et al.*, 2014). In the respective study 25% (9/35) patients fell between the grey zone of > 1E+04 and 1E+06 with authors stating that these patients needed to be assessed on a case by case basis to determine the significance of the positive result (Maillet *et al.*, 2014). In the respective study the threshold for colonization was also high at <3160 copies/mL, which is slightly higher that the published LOD for IF microscopy.

In the majority of published studies a patient with >1 x $10^3$ cfu/mL would be considered to have probable PCP. In study 3 (table 8.12) Alanio and colleagues defined thresholds for colonisation and infection utilising a multi-copy gene MitLSU and converting the results to trophic form equivalents (TFE) for interpretations (Alanio, Desoubeaux, *et al.*, 2011). This approach is likely to be most accurate as it not only quantifies the amount of target being detected but also the standards are extracted through the same workflow as the clinical samples and thus any loss of DNA through extraction occurs for both the standards and samples. A threshold of <190 TFE/mL defined colonisation verses a threshold of >1.9E+4 TFE defining infection (Alanio, Desoubeaux, *et al.*, 2011). Alanio reported 3.9% of patient for which fungal load was in the grey zone (≥120 and ≤1900 TFEq/mL), and the infection status of the patient could not be determined. Studies 4 and 5 also set thresholds similar to that of Alanio *et al* with the colonisation thresholds close to the 100 cells/mL target copy per/mL mark and infection at >$10^3$ cells/mL. Chumpitazi and colleagues chose to utilise a low threshold of 54 copies/mL

for discriminating colonisation with infection, however this study had a high prevalence of 27.3% (18/66) which may have biased the calculated performance of the PCR, dampening the effects of false positive results (Chumpitazi *et al.*, 2011) (table 8.12). However, there is disparity between thresholds set across studies utilising qPCR. In 80% (4/5) of the studies in table 8.12 the PPV was >95% suggesting that thresholds do increase the performance of PCR for the diagnosis of PCP.

**Table 8.12 *P. jirovecii* qPCR studies alongside published thresholds to define infection/colonization and performance characteristics.**

| Study | Author | Target (copy) | Threshold | Cells /mL | PPV | NPV | Sens | Spec |
|---|---|---|---|---|---|---|---|---|
| 1 | Maillet | MSG (single) | <3160 tc/mL<br>>31600 tc/mL | 3E+04<br>3E+06 | -<br>100 | 100<br>- | 100<br>- | 70<br>80 |
| 2 | Chumpitazi | MSG (single) | 54.3 t/mL | 5.4E+01 | 95.5 | 100 | 100 | 97.7 |
| 3 | Alanio | LmitSU (100-1000) | <120 TFE/mL<br>>1900 TFE/mL | 1.2E+02<br>1.9E+03 | -<br>100 | 100<br>- | 100<br>85.7 | 96.9<br>100 |
| 4 | Matsumara | DHPS (single) | <340 tc/mL<br>>1300 tc/mL | 3.4 E+02<br>1.3E+03 | 78.6 | 100 | 100 | 80.0 |
| 5 | Muhlethaler | Unknown (single) | <85 tc/mL<br>>1450 tc/mL | 8.5E+01<br>1.5E+03 | -<br>98 | 100<br>- | 100<br>- | 99.4<br>- |
| 6 | Rudramurthy | MSG (single) | CT ≤25<br>CT 26-36 | -<br>- | 100 | 100 | 100 | 100 |

In our study the application of thresholds to define infection from colonisation would have led to the exclusion of one patient with proven disease with a low positive at 1.5E+1-1.5E+2 cells/mL and would not have resolved the false positive result tin a control patient whose fungal burden was determined to be 8.2 E+4-8.2 E+5 cells/mL. Both respective patients were not textbook HIV infected PCP cases and this may be the reason for the disparity in performance of PCR in our study.

The use of thresholds for the interpretation of qPCR results in HIV infected patients is more successful that in non-HIV infected patients. In 2008, Hugget and colleagues applied qPCR specifically to the detection of *P. jirovecii* from BAL in HIV infected patients. The respective study reported 98% sensitivity and 96% specificity for the diagnosis of PCP when using a threshold of >10 copies per reaction, which would have equated to approximately 60-120 cfu/mL (Huggett *et al.*, 2008). Teh *et al* reported no clear relationship between qPCR results and clinical features or outcome in the non-HIV patient cohort and concluded that qualitative PCR and using a negative result to exclude PCP was most appropriate (Teh *et al.*, 2014). Furthermore, it was concluded

that positive results should be interpreted in the clinical context of the patient rather than using thresholds to define their significance. This finding is supported by further studies (Mühlethaler *et al.*, 2012; Seah *et al.*, 2012a; Robert-Gangneux *et al.*, 2014) and by the findings of a meta-analysis that 31% of patients labelled with false positive PCR results had or later developed PCP infection (Lu *et al.*, 2011).

From this study it is clear that the close interaction between the laboratory and clinicians in the provision of a molecular diagnostic service for PCP is crucial. Results need to be interpreted on a case-by-case basis. The introduction of a new molecular diagnostic assay also needs to be appropriately justified; sufficient numbers of samples are needed to render it cost effective and also to ensure that the laboratory maintains the expertise in interpreting the results. For these reasons National reference laboratories should be considered, as the testing provider if the number of samples expected to be tested is not significant. Services may be brought in-house following a period of parallel testing using specialist referral labs as a benchmark. As induced sputum and BAL are acquired through moderately invasive poorly tolerated procedures a high clinical suspicion of infection is required to justify collecting these specimens and not every patient ill be suitable for these procedures. Therefore relatively few samples may be submitted for investigation to an individual laboratory, but for larger regional or national reference laboratories serving several hospitals the use of PCR is more strongly justified. As the incidence of PCP remains low in the HIV population following the advent of anti-retroviral treatment and in the non-HIV patient population due to prophylaxis the implementation of a PCR for *P. jirovecii* as a diagnostic assay requires consideration not only in terms of the assay but in the wider context of the patient population which in turn influences the performance of the test and how it is interpreted and reported to the clinical interface. This study has demonstrated that PCP PCR can demonstrate a high NPV and specificity for the exclusion of PCP in at risk patients. Further developments in standardising PCR and defining thresholds for interpretation are required to achieve a robust positive predicative value that will allow clinicians to have the confidence to diagnose disease with accuracy.

# Chapter 9 Evaluation of PCR for screening patients with haematological malignancy for the detection of Invasive Aspergillosis (IA)

## 9.1 Introduction

The detection and diagnosis of invasive aspergillosis relies heavily on radiological imaging by high resolution computer tomography (HRCT) which has a sensitivity of 96% and specificity of 65.4% respectively for the diagnosis of invasive aspergillosis (IA) (Prem *et al.*, 2010) if the characteristic features, including lung nodules with ground glass/halo sign are detected (Escuissato *et al.*, 2005; Gasparetto, Escuissato and Marchiori, 2008; Qin *et al.*, 2012). The laboratory's role in the diagnostic pathway includes provision of an enzyme immune assay (EIA) that detects circulating Galactomannan enzyme immuno assay (GM-EIA). Galactomannan is a cell wall lipopolysaccharide of *Aspergillus* species (Bernard and Latgé, 2001) which serves as a cell wall storage polysaccharide but is also a powerful antigen excreted during fungal growth (Engel, Schmalhorst and Routier, 2012; Free, 2013). Culture from clinical specimens also has some but limited utility. Biopsies for histopathological analysis with fungal specific stains are the current gold standard for diagnosis of invasive infection. The respective tests, including HRCT and GM-EIA are included in the European Organization for Research and Treatment of Cancer (EORTC) Mycoses Study Group guidelines for defining invasive fungal disease (IFD) (De Pauw *et al.*, 2008).

In recent years *Aspergillus* PCR has been investigated for its utility as a screening tool for the detection of invasive aspergillosis. Serological specimens are favoured when screening, as high frequency sampling is required. The specimen types most commonly analysed being whole blood (Bernal-Martínez *et al.*, 2011; Löffler and Kurzai, 2011; Springer *et al.*, 2013) and serum, with improved performance achieved when analysing plasma (J. Loeffler *et al.*, 2000; White *et al.*, 2015). The most suitable fraction of blood has not definitively been determined to date. A recent meta-analysis reported a combined sensitivity of 84% (95% CI 75, 91) and a specificity of 76% (95% CI 65, 84) for the performance of PCR in the diagnosis of invasive aspergillosis (Arvanitis, Anagnostou and Mylonakis, 2015) with negative PCR results used to exclude disease as the combined negative predictive value across 25 studies in this analysis was 96% (Arvanitis, Anagnostou and Mylonakis, 2015). However, positive results cannot be used to definitively diagnose disease, as the combined PPV was reported to be only 38% (Arvanitis, Anagnostou and Mylonakis, 2015). The

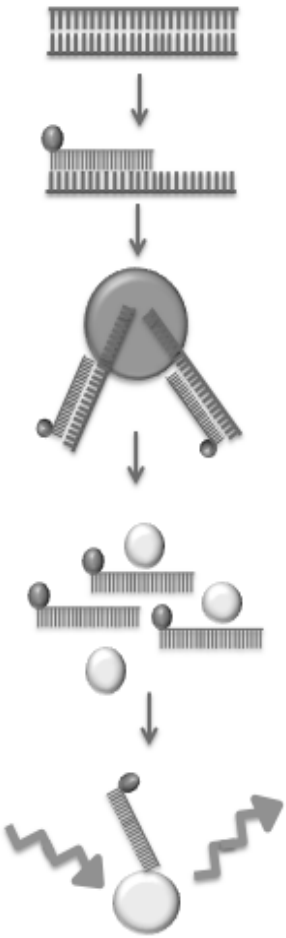
interpretation of positive results remains the on-going challenge as false positive results are frequently encountered, most likely a result of environmental contamination or a lack of specificity in the PCR assays being utilized (Alanio, Desoubeaux, *et al.*, 2011). Recent studies have demonstrated that combining PCR with antigen testing (Morrissey *et al.*, 2013; Rogers *et al.*, 2013; Aguado *et al.*, 2015) improves the performance of biomarker detection for the diagnosis of IA and may also enable earlier detection of disease (Morrissey *et al.*, 2013; Rogers *et al.*, 2013; Aguado *et al.*, 2015).

With evidence pointing to the use of PCR as one of the primary methods to support the diagnosis of invasive aspergillosis there is a question as to why guidelines to date have not recommended its use. One of the major factors prohibiting the introduction of PCR across laboratories globally for diagnosis of IA is a lack of a standardisation across the molecular method employed (De Pauw *et al.*, 2008). Over the last 10 years a multi-centre collaboration, the European *Aspergillus* PCR Initiative (EAPCRI) has worked to identify the critical steps in standardizing the performance of *Aspergillus* PCR from serological and respiratory specimens (White *et al.*, 2010). The aim was to better understand critical factors in the application of PCR in this setting and how best to utilize PCR alongside other diagnostic techniques to improve diagnosis. Studies from the EAPCRI group have highlighted the importance of the nucleic acid extraction protocol in achieving satisfactory analytical sensitivity (White *et al.*, 2010). Input sample volume, elution volume and mechanical lysis when extracting from whole blood were also critical (White *et al.*, 2010). As DNA extraction from whole blood yields variable results serum has emerged as a more optimal sample type for *Aspergillus* PCR, and facilitates dual biomarker testing as it is the sample type also used in GM-EIA analysis (White *et al.*, 2010). When considering the performance of *Aspergillus* PCR a mean sensitivity and specificity of 71.8% and 96% were reported from five studies (Bretagne *et al.*, 1998; Williamson *et al.*, 2000; Costa *et al.*, 2002; Pham *et al.*, 2003; Halliday *et al.*, 2006).

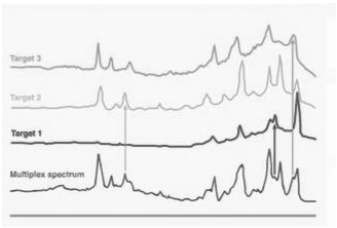
The majority of studies have utilized in-house developed *Aspergillus* PCR assays and in our centre the 28Asp assay, a PCR targeting the 28S ribosomal RNA gene, utilized in the EAPCRI studies is the assay of choice (White *et al.*, 2010, 2014; Löffler and Kurzai, 2011). As highlighted in previous chapters the use of in house assays can contribute to varied performance across centers (Walker and Subasinghe, 1999; Burkardt, 2000; Techniques, 2000) centres. One solution is to utilize commercially validated assays, which assist with standardizing processes across centres, allowing for standardised laboratory methodologies to generate data to compare both disease

prevalence and on-going performance. Commercial assays also have the benefit of manufacturing and production quality control to national and international manufacturing standards. Although in-house assays can be adequately quality controlled, often using high yield clinical samples, what they are unable to do as material is exhausted is to provide longitudinal quality control over time to monitor for assay drift. This is a crucial factor when assessing both assay performance and disease prevalence over long periods. At the time of undertaking this research a new commercial assay for the detection of *Aspergillus* from clinical specimens by PCR had been developed for diagnostic use. This assay employed a novel approach coupling conventional PCR with probe based chemistry and Raman scattering spectra (SERS) (White *et al.*, 2014). SERS utilizes sensitive spectroscopic detection to generate analyte-specific fingerprint spectra (White *et al.*, 2014), Figure 9.1.

Raman spectroscopy is a detection method that captures molecular vibrations as a mass spectrum and is used in many applications for identification and quantitation. The vibrations, or Raman scattering, are strongly enhanced as a molecule is bound to a metallic surface (Stiles *et al.*, 2008; Schlücker, 2014). In the case of the fungiplex the molecule is the species-specific probe and the metalic surface is the metallic capture beads. As the electromagnetic wave in the Raman spectroscope interacts with the metal surface plasmons, coherent delocalized electron oscillations that exist at the interface between any two materials, are excited (Stiles *et al.*, 2008; Schlücker, 2014). The magnitude of the electromagnetic fields at the surface is enhanced if the species-specific probe has adsorbed onto the metal bead. The electronic coupling between the metal and the molecule increases its Raman-scattering signal which is then detected (Stiles *et al.*, 2008; Schlücker, 2014). In the case of the Fungiplex assay a positive PCR will result in probe being adsorbed onto the metal beads whereas a negative reaction will only warrant beads with no bound molecule and therefore no Raman signal. The method is also claimed to be more sensitive than standard real time PCR applications making it suitable for its application in the IA setting (White *et al.*, 2014). The aim of this study was to compare an EAPCRI compliant 28Asp real time PCR with the RenDX Fungiplex® assay for the detection of *Aspergillus* DNA from serum as a screening approach for the detection of IA in haemato oncology patients.



1. PCR amplification of extracted DNA.
2. Specific RenDX probes are hybridized to biotinylated target DNA.
3. Hybridised DNA is captured on streptavidin bound magnetic beads
4. Probes eluted from captured DNA
5. Eluted probes are adsorbed onto silver nano particles and analysed.
6. Sample is exposed to a Raman laser and a detector receives the Raman signal reflected off the sample.




7. SERS signal detailing an example base line spectrum for a negative sample (lowest line) with positive target signals in the upper three lines showing additional peaks for the associated bound probes.

Figure 9.1 Schematic of PCR-SERS amplification and detection.

## 9.2 Methods

### 9.2.1 Clinical Samples

Sera submitted to the laboratory for the investigation of fungal infection, including specifically GM-ELISA testing, from patients undergoing chemotherapy for acute myeloid leukaemia (AML), myelodysplastic syndrome, or allogeneic stem cell transplantation and receiving itraconazole prophylaxis were included. The samples were tested under the umbrella of service development, whereby specimens can be utilised for the development of diagnostics tests directly relating to the diagnosis of disease that the specimen was originally submitted for, in this case galactomannan testing for the investigation of Aspergillosis. Disease status was classified using EORTC/MSG (2008) criteria (de Pauw *et al.*, 2008). Table 9.1 shows the EORTC/MSG criteria used to classify patients. Those with probable disease require one factor from all three categories including: host, clinical and mycological categories. For possible disease the mycological criteria is omitted as being essential.

**Table 9.1 EORTC/MSG criteria for defining probable and possible Invasive Fungal Disease.**

| | |
|---|---|
| Host factors | History of neutropenia (<500 neutrophils/$mm^3$ for >10 days) related to the onset of fungal disease. |
| | Receipt of an allogeneic stem cell transplant. |
| | Prolonged use of corticosteroids. |
| | Treatment with other recognized T cell immunosuppressant, such as cyclosporine. |
| | Inherited severe immunodeficiency (such as chronic granulomatous disease). |
| Clinical criteria | The presence of 1 of the following 3 signs on CT: |
| | Dense, well-circumscribed lesions(s) |
| | With or without a halo sign, air-crescent sign or cavity |
| Mycological criteria | Direct microscopy positive for hyphae from BA or sputum. |
| | Galactomannan antigen detected in serum or bronchoalveolar lavage fluid. |
| | Recovery, by culture, of *Aspergillus* from BAL or sputum. |

### 9.2.2 Culture of *Aspergillus fumigatus* control strain

*Aspergillus* control strain *Aspergillus* fumigatus ATCC® 1022™ Designation: NRRL 163 was sub cultured onto Sabouraud's agar, incubated for 2-3 days until colonies had developed with fruiting structures. The control strain was then cultured as mycelial mats for DNA extraction as detailed in section 2.1.4

### 9.2.3 Extraction of DNA from *Aspergillus fumigatus* control strain;

See general methods 2.2. In addition extracted DNA was quantified using the Nanodrop spectrophotometer and dilutions of genomic DNA were prepared in 1 x (Tris Ethylenediaminetetraacetic acid (EDTA) buffer.

### 9.2.4 Extraction of genomic DNA from potato (IC SPUD)

Internal control methodology was adapted from Honeybourne *et al* (2011). 1cm$^2$ sections of *Solanum tuberosum* (potato) tuber were snap frozen in liquid nitrogen and ground to powder using a pestle and mortar. Total genomic DNA was extracted from 100mg of this powder using the GenElute Plant Genomic DNA Miniprep kit, Sigma according to the manufacturer's instructions.

### 9.2.5 Generating the SPUD DNA IC control

A 1957 bp fragment of the tuber gene was amplified using forward primer 5'-ggttcactcacaatcaaagcattcaaaccc-3' (Honeyborne *et al.*, 2011) and reverse primer 5'-ggaagctgtgggtggcggtcgaaattcaa primers3' (Honeyborne *et al.*, 2011). The PCR amplicon was verified as being the correct tuber gene fragment by sequence analysis, see general methods 2.2. The product was purified by gel extraction (QIA*Quick* Gel Extraction kit, Qiagen, UK) see general methods 2.2 and then quantified using a Nanodrop Spectrophotometer (ThermoFisher, USA) see general methods 2.2.

### 9.2.6 DNA extraction from serum

DNA was extracted from 1mL of serum through the Diasorin IXT automated workflow as per manufacturer's instructions (Diasorin, Ireland). Viral nucleic acid cartridges were used to extract DNA from serum. Samples were loaded onto the platform, 10µL proteinase K was added and an internal control (10µL 0.002ng SPUD) and the large volume extraction protocol was selected, with a run time of approximately 45 minutes. The extracted nucleic acid was eluted into a final volume of 50µL of eluate, in line with EAPCRI recommendations (White *et al.*, 2010). A negative extraction control was

included in every run. A maximum number of 11 samples per extraction run were possible.

### 9.2.7 28Asp real time PCR

A previously published *Aspergillus* real-time PCR (28Asp) targeting the 28S rRNA gene was utilized (White *et al.*, 2010). Primers and probes sequences were as follows; forward primer ASF1 5'-GCA CGT GAA ATT GTT GAA AGG-3', reverse primer ADR1 5'-CAG GCT GGC CGC ATT G-3' and Taqman probe ASP 28P 5'-FAM- CAT TCG TGC CGG TGT ACT TCC CCG-TAMARA-3'. For the inhibition control SPUD PCR forward primer IC F 5'-GCACAGGGTTGATGTTGGTATTGTC-3' Tm 63°C, reverse primer IC R 5'-CAAATGAGAAATAGCCCTCACTGCAAG-3' Tm 63.4°C and probe IC Probe 5'-JOE-GCAGGGTCCTCAGTTCTAGCAGGCTCCA-BHQ1-3' were utilized. *Aspergillus* PCR reactions were run in duplicate with separate internal control reaction in third tube.

The assay was performed for 45 cycles as a single-round assay using a Rotorgene Q HRM instrument (Qiagen, United Kingdom). The final reaction composition in a 50 µL, reaction contained 0.75µM primers, 0.4µM hydrolysis probe, 4mM $MgCl_5$, Abgene QPCR 2 x master mix and 10µL template DNA. PCR plasmid controls were also utilized to allow for run verification at 500, 50 and 5 input copies per reaction. For the 28Asp PCR expected $C_T$ values for 300 plasmid copies was 32, 30 plasmid copies 35 and 3 plasmid copies 39. Control plasmid was supplied by Dr P White, UKCMN Regional Mycology Reference Laboratory PHW Microbiology Cardiff Heath Park, Cardiff.

### 9.2.8 PCR amplification and SERS detection

PCR-SERS testing was performed, according to the manufacturer's instructions. The first stage of the assay was a conventional PCR in a 50µL reaction to which 10µL of DNA template was added. The PCR assay targets the 28S rRNA genes, generating an amplicon of approximately 200bp in size. A negative test control (NTC) of molecular grade water was included in each PCR run and processed from extraction through to PCR. An internal amplification control was added prior to extract the amplification process. Once the PCR amplification was complete the second stage of processing was SERS probe hybridisation. Post amplification steps included transferring 5µL of the amplified product to a 96 well microtitre plate containing 15µL of SERS detection probe. The probe/amplicon mix was heated to 95°C for 15 minutes to allow for

denaturing, annealing and hybridisation. Once complete the hybridised products were processed on a RenDX (Renishaw Diagnostics, UK) sample processor where samples were passed through a series of wash buffers. Remaining bound probe was released into the elution buffer in preparation for detection on the SERS platform. Figure 9.1 details the process of Fungiplex SERS detection and resulting SERS trace from which a result is interpreted.

The total process, including DNA extraction, was completed within one working day (8hours), with the PCR amplification-SERS analysis taking approximately 6hours to complete. Operator hands-on time of approximately 2 hours including DNA extraction was needed. The Raman spectroscopy instrument software, using a direct classical least-squares model of analysis, automatically interpreted results as positive, negative, or inhibited.

### 9.2.9 Determination of the analytical sensitivity of the 28ASP real time PCR and Fungiplex workflow;

Serial dilutions of plasmid DNA containing the 28Asp target were processed through the 28Asp PCR, in eight replicates, to assess the analytical sensitivity and reproducibility at an input of 2000, 200, 20 and 2 plasmids per reaction.

*A. fumigatus* genomic DNA sample was diluted into high medium and low concentrations.

a) 10µL of each dilution was processed through each PCR in 12 replicates.

b) 20µL of each *A. fumigatus* genomic DNA dilution was spiked into 2mL serum samples in six replicates and extraction performed from 550µL and 1mL of the spiked serum.

The representative copy number of the *A. fumigatus* genomic DNA was calculated by quantifying the results against a standard curve generated using the control plasmid through the 28Asp PCR.

### 9.2.10 Assessing the clinical performance of the 28ASP real time PCR and Fungiplex workflow

Patients were classified into proven, probable or control groups through analysis of radiological, laboratory and clinical investigations according to criteria described in table 9.1. Classifications were reviewed by two operators, firstly by a senior scientist and secondly by a clinical consultant mycologist. Serum submitted for GM-EIA testing was processed through DNA extraction and tested using the 28Asp and Fungiplex in parallel. Samples were batched into a minimum of 20 samples processed at one time to allow for more efficient processing. The investigators were blinded to the EIA testing results, which were collected retrospectively after the completion of PCR processing of the complete sample set.

### 9.2.11 Statistical analysis

Clinical performance was assessed by calculating sensitivity, specificity, positive and negative predictive values for both PCR assays versus the EORTC definitions assigned to each patient. Two by two binary concordance tables were constructed, using both probable IFD and probable/possible IFD as true cases and NEF patients as the control population. A single positive PCR reaction was required to consider a patient positive The agreement between the Fungiplex assay and the original 28Asp real-time PCR results was determined by calculating the kappa statistic and observed agreement. Receiver operating characteristic (ROC) curves were generated using the earliest $C_T$ value generated per patient to determine whether a particular $C_T$ threshold provided optimal PCR performance for the 28Asp assay. For this analysis in patients negative by PCR, a $C_T$ value of 41 cycles was applied, representing the lowest number of PCR cycles performed.

## 9.3 Results

### 9.3.1 Analytical sensitivity and performance of the 28Asp PCR;

Table 9.2 details median $C_T$ values of 26.1(IQR 25.9, 26.2) for 2000 copies, 30.7 (IQR: 30.6, 31.1) for 200 copies, 33.9 (IQR: 33.7, 34.2) for 20 copies and 38.9 (IQR: 38.2, 39.4) for 2 copies per reaction with 100% (10/10) reproducibility across all dilutions. Variance in $C_T$ values increased slightly for the 2 plasmid copies per reaction dilution, figure 9.2, but was not significantly different across any of the plasmid dilutions.

The calculated efficiency of the 28Asp PCR was 74% (slope -4.16).

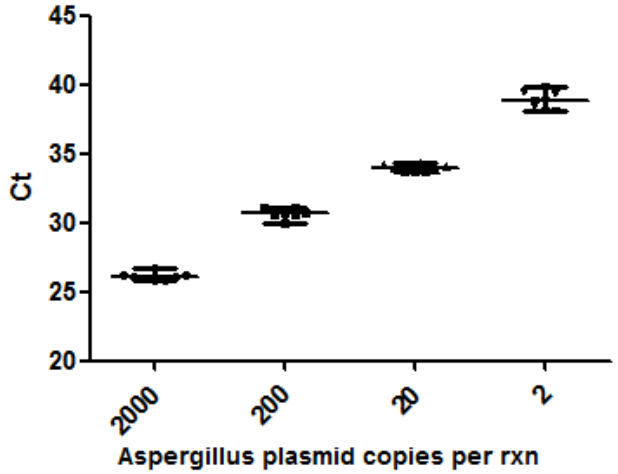



Figure 9.2 $C_T$ values for plasmid dilutions processed through the 28Asp PCR, with median values and IQR indicated.

**Table 9.2 Reproducibility of 28Asp PCR across serial dilutions of plasmid target with median $C_T$ values and IQR presented.**

| Plasmid copies per reaction | % Reproducibility | Median $C_T$ | IQR |
|---|---|---|---|
| 2000 | 100% (8/8) | 26.1 | 25.9, 26.2 |
| 200 | 100% (8/8) | 30.7 | 30.6, 31.1 |
| 20 | 100% (8/8) | 33.9 | 33.7, 34.2 |
| 2 | 100% (8/8) | 38.9 | 38.2, 39.4 |

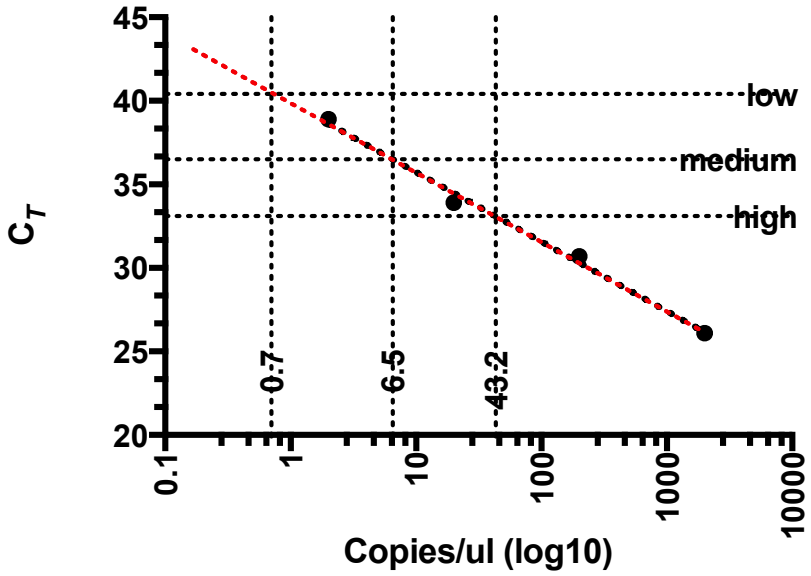



Figure 9.3 Standard curve derived from target plasmid dilutions processed through the 28Asp PCR for which the high, medium and low genomic DNA dilutions were measured against to estimate the equivalent plasmid copies.

### 9.3.2 Characterizing Genomic DNA dilutions

Equivalent plasmid copies for each genomic DNA dilution were calculated by testing 12 replicates of each DNA dilution through the 28Asp PCR and measuring the $C_T$ result against a standard curve generated from plasmid dilutions, see figure 9.3 and table 9.3. The high genomic concentration of approximately 1pg/µL was equivalent to $C_T$ 43.2 (95% CI: 31.1 to 57.9) plasmid copies input, the medium DNA concentration of approximately 100fg/µL was equivalent to $C_T$ 6.5 (95% CI: 2.8 to 14.4) plasmid copies and the low DNA concentration of approximately 10fg/µL was equivalent to $C_T$ 0.74 (95% CI: 0.03 to 5.6) plasmid copies input per reaction, see figure 9.3.

**Table 9.3 Equivalent plasmid copies per mL for *A. fumigatus* genomic DNA dilutions.**

| Plasmid copies per reaction | % reproducibility | Median $C_T$ | PC eq | 95%CI |
|---|---|---|---|---|
| High | 100% (12/12) | 33.1 | 43.2 | 31.1, 57.9 |
| Medium | 100% (12/12) | 36.5 | 6.5 | 2.8, 14.4 |
| Low | 62.5% (5/12) | 40.4 | 0.7 | 0.03, 5.6 |

**PC eq = plasmid copy equivalent**

### 9.3.3 Analytical performance of the Fungiplex PCR-SERS assay compared with the 28Asp real time PCR assay

100% (12/12 95%CI: 70.1, 100) reproducibility was observed through each PCR for the detection of the high and medium DNA dilutions. For the low concentration of genomic DNA reproducibility was 55.6% (5/9, 95%CI: 26.7, 81.1) for the Fungiplex PCR SERS assay and 66.7% (6/9, 95%CI: 35.42, 87.94) reproducibility for the 28Asp qPCR, table 9.4.

**Table 9.4 Reproducibility of detection of the Fungiplex PCR-SERS assay and 28Asp PCR for high, medium and low genomic DNA dilutions processed directly through each PCR.**

| | **High 1pg/µL (43.2 Pc)** | | **Medium 100fg/µL (6.5 Pc)** | | **Low 10fg/µL (0.7 Pc)** | |
|---|---|---|---|---|---|---|
| | % (95% CI) | *n* | % (95% CI) | *n* | % (95% CI) | *N* |
| **Fungiplex** | 100% (70.1, 100) | 12/12 | 100% (70.1, 100) | 12/12 | 55.6% (26.7, 81.1) | 5/12 |
| **28Asp qPCR** | 100% (70.1, 100) | 12/12 | 100% (70.1, 100) | 12/12 | 66.7% (35.4, 87.9) | 6/12 |

### 9.3.4 Determination of the limit of detection of the complete workflow; extraction to PCR detection

When analysing extracts from 1mL of serum spiked with the high and medium concentrations of genomic DNA both the 28Asp and Fungiplex PCR detected *A. fumigatus* from 100% (6/6) of the extracts with a mean $C_T$ values of 33.1 (33.7-35.2) and 38.6 (37.6-39) respectively for the 28Asp PCR. When analysing extracts from 1mL of serum spiked with the low concentration of genomic DNA (representing 0.7 plasmid copies/uL) 83.3% (5/6) of the extracts were positive for *A. fumigatus* by the 28ASP PCR with a mean $C_T$ of 40 (38.5-41.4) and 50% (3/6) for the Fungiplex PCR, table 9.5.

When analysing extracts from 550µL of serum both the 28ASP, with a median $C_T$ of 35.8 (34.8-36.5) and Fungiplex PCR detected *A. fumigatus* from 100% of the extracts. For the medium spiked serum the 28ASP PCR detected *A. fumigatus* for 66.7% (4/6) of the extracts with a mean $C_T$ of 39.3 (38.9-39.7) and the Fungiplex from 83.3% (5/6) of the extracts. For the low spiked serum the 28ASP PCR was positive from a single sample with a $C_T$ of 40.5, whereas the Fungiplex failed to detect *A. fumigatus* from all 6 extracts, table 9.5.

**Table 9.5 $C_T$ values and reproducibility of 28Asp PCR and Fungiplex for the detection of *Aspergillus fumigatus* genomic DNA extracted from 550µL and 1mL of serum on the Diasorin IXT platform compared with direct PCR.**

| | Direct | | | | 1mL | | | | 550µL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 28ASP PCR | | | RENDX PCR | 28ASP PCR | | | RENDX PCR | 28Asp PCR | | | RENDX PCR |
| | $C_T$ | Dynamic Range | Reproducibility % | | $C_T$ | Dynamic Range | Reproducibility % | | $C_T$ | Dynamic Range | Reproducibility % | |
| High (43.2 Pc/µL) | 33.1 | 31.4-35.7 | 100 (12/12) | 100 (12/12) | 34.3 | 33.7-35.2 | 100 6/6) | 100 6/6) | 35.8 | 34.8-36.5 | 100 (6/6) | 100 (6/6) |
| Med (6.5 Pc/µL) | 36.5 | 33.6-37.8 | 100 (12/12) | 100 (12/12) | 38.6 | 37.6-39 | 100 (6/6) | 100 (6/6) | 39.3 | 38.9-39.7 | 66.7 (4/6) | 83.3 (5/6) |
| Low 0.7 Pc/µL) | 40.4 | 38.2-41.3 | 41.7 (5/12) | 50 (6/12) | 40 | 38.5-41.4 | 83.3 (5/6) | 50 (3/6) | 40.5 | - | 13 (1/6) | 0 (0/6) |

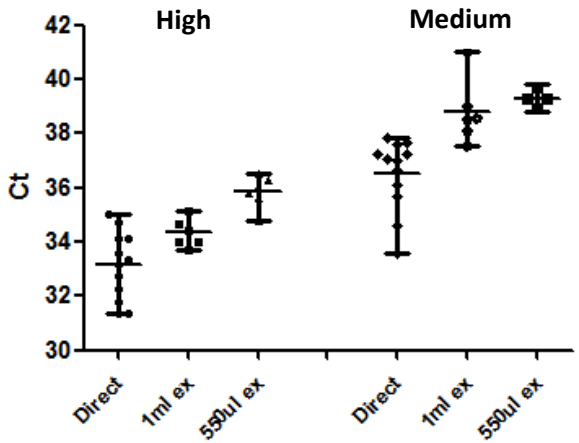



Figure 9.4 demonstrates $C_T$ values for the *A. fumigatus* high and medium genomic DNA dilutions directly spiked into the 28Asp PCR assay, extracted from 1mL and 550µL of serum, demonstrating the loss in sensitivity when extracting DNA from lower volumes of serum.

Figure 9.4 demonstrates a scatter plot of $C_T$ values, with the median and IQR ranges shown, for the high and medium *A. fumigatus* genomic DNA concentrations processed through the 28Asp PCR. There was no significant difference in $C_T$ values when testing the high concentration directly spiked into the PCR and when extracted from 1mL of serum ($p<0.09$), whereas when the high concentration was extracted from 550µL of serum the $C_T$ values were significantly higher than the direct and 1mL analysis. When testing the medium concentration of *A. fumigatus* genomic DNA the $C_T$ values were significantly higher than the direct analysis ($p<0.01$), but no significant difference ($p=0.07$) was observed in the $C_T$ values when extracting from 1mL and 550µL volumes of serum for the medium concentration of genomic DNA, figure 9.4.

### 9.3.5 Clinical performance of the 28Asp and Fungiplex PCR

Utilising methodological data generated from previous analytical investigations, analysis of clinical specimens was performed from 1mL of serum, as this specimen volume had been previously shown to provide optimal performance through both PCR assays.

**Patient demographics**; in total 83 patients were screened for fungal infection during the time frame of this study. Table 9.6 demonstrates the demographics for patients included. The median age was 52 years (range 24-85). Of the underlying haematological conditions acute myeloid leukaemia (AML) accounted for 51.8% (43/83), acute lymphoblastic leukaemia (ALL) accounted for 18.1% (15/82). Lymphoma accounted for 16.9% (14/83) and Myeloma 9.6% (8/83). Two patients were categorised in the other haematological malignancy subgroups, with one patient diagnosed with a RAEB1 with monosomy 7 immunodeficiency and the second with Wiskott - Aldrich syndrome.

| Table 9.6. Patient demographics with EORTC/MSG definitions of IFD. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **Range** | | | **M** | **F** | | |
| **Age** | 52 | 24-85 | **Sex** | | 58% | 42% | | |
| | **EORTC definition** | | | | | | | |
| | **Probable (2)** | | **Possible (11)** | | **NEF (70)** | | **Overall** | |
| **Underlying condition** | **%** | **n** | **%** | ***n*** | **%** | ***n*** | **%** | ***n*** |
| ALL | | | 1.2 | 1 | 16.9 | 14 | 18.1 | 15 |
| AML | 2.4 | 2 | 8.4 | 5 | 43.4 | 36 | 51.8 | 43 |
| CLL | | | | | 1.2 | 1 | 1.2 | 1 |
| Myeloma/lymphoma | | | | 2 | 24.1 | 20 | 26.5 | 22 |
| Other | | | | | 2.4 | 2 | 2.4 | 2 |
| HRCT | 2.4 | 2 | 13.3 | 11 | 32.5 | 27 | 51.8 | 43 |

NEF = no evidence of fungal disease

### 9.3.6 Screening characteristics

In total 343 samples were submitted to the laboratory for investigation, Sample frequencies sent during neutropenic episodes were 1 sample for 25.3% (22/83) patients, 2.4% (2/83) samples for 18.1% (15/83) patients, 3 samples for 20.5 (17/83) patients and ≥4 samples for 48.2% (30/83) patients.

#### 9.3.6.1 Classification of disease status according to EORTC criteria

From the cohort of 83 patients none were classified as having proven invasive fungal disease.

#### 9.3.6.2 Control patients 'No evidence of fungal disease 'NEF'

Seventy-three patients were classified as having no evidence of fungal infection on HRCT 32.5% (27/83) or did not undergo HRCT scanning, as fungal infection was not clinically suspected 51.8% (43/83).

#### 9.3.6.3 Possible invasive fungal disease

Eight patients were classified as having a possible fungal disease. HRCT demonstrated pulmonary lesions consistent with invasive aspergillosis described as defined nodules and ground glass shadowing. All 8 patients had a period of profound neutropenia >10 days meeting the clinical criterion required for possible infection. For

all 8 patients GM EIA analysis was negative and cultures from clinical specimens did not grow *Aspergillus sp*. Of note 50% (4/8) patients had >4 samples submitted for GM EIA during their neutropenic episode. The remaining 50% (4/8) had 2 (2) or 3 (2) samples submitted for screening.

#### 9.3.6.4 Probable invasive fungal disease

Two patients were classified as having probable invasive fungal disease with HRCT features consistent with invasive aspergillosis, a neutropenic episode >10 days but also mycological criterion of a positive GM EIA during their neutropenic episode. Patient 1 had a single sample submitted for GM EIA analysis with an index of 2.184, which on repeat measured at 0.84. Patient 2 had 10 samples submitted for GM EIA analysis. One sample submitted for patient 2 was positive measuring an index value of 0.989 and 0.86 on repeat, two equivocal samples measuring 0.516 and 0.636 on initial testing but on repeat measured 0.11 and 0.10 and 7 samples all measuring negative on initial testing with index values <0.5.

### 9.3.7 Performance of Galactomannan EIA for the detection of IA

The overall performance of GM EIA for probable patients was a sensitivity of 100% (32.2, 100), specificity of 97.5% (91.3, 99.3), PPV of 50% (15, 85) and NPV of 100% (95.3, 100). Overall diagnostic accuracy was 97.6% (91.5, 99.3). Performance of GM EIA in probable/possible patients had a sensitivity of 22.2% (6.3, 54.7), specificity of 97.3% (90.7, 99.3, PPV of 50% (15, 85) and NPV of 91.1% (82.8, 95.6). Overall diagnostic accuracy was 89.2% (80.7, 94.2).

Table 9.7 details the four patients with positive GM EIA results. Patient 1 with probable IA had two equivocal GM results measuring 0.636/0.11 and 0.516/0.10 and one positive result measuring 0.99/0.86 over a four-month neutropenic period. Patient 2 with probable IA had a single sample submitted to the laboratory, which was positive by GM EIA, measuring 2.184/0.82. Patients 3 had a positive GM EIA result for the only sample submitted to the laboratory measuring 3.744/1.60, but was not suspected to have IA and no HRCT was not requested on this patient. The final Patient 4 had four samples submitted to the laboratory, with three positive GM EIA results in succession 0.853/0.675, 0.86/1.271 and 1.01/0.64 however, the HRCT demonstrated no evidence of invasive aspergillosis.

**Table 9.7 Patients positive by GM EIA with corresponding PCR results for the 28Asp and the Fungiplex assay.**

| Patient | Underlying Disease | Radiology HRCT | EORTC | Sample date | GMI 1 | GMI 2 | GM EIA Result | 28Asp (1) | 28Asp (2) | 28Asp Result | Fungiplex Result |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | AML | Positive | Probable | 10/04/14 | 0.636 | 0.11 | Equivocal | 38.43 | 36.61 | *Positive* | Negative |
| | | | | 14/04/14 | NT | - | NT | 40.83 | 40.91 | *Positive* | Negative |
| | | | | 17/04/14 | 0.12 | - | Negative | 0 | 0 | Negative | Negative |
| | | | | 21/04/14 | 0.13 | - | Negative | 40.29 | 0 | PNC | Negative |
| | | | | 26/05/14 | 0.10 | - | Negative | 0 | 0 | Negative | Negative |
| | | | | 09/06/14 | 0.989 | 0.86 | Positive | 37.93 | 40.52 | *Positive* | Negative |
| | | | | 14/06/14 | NT | - | NT | 39.91 | 40.25 | *Positive* | Negative |
| | | | | 21/07/14 | 0.516 | 0.10 | Equivocal | 0 | 0 | Negative | Negative |
| | | | | 31/07/14 | 0.16 | - | Negative | 0 | 0 | Negative | Negative |
| | | | | 13/08/14 | 0.18 | - | Negative | 40.15 | 41.5 | *Positive* | *Positive* |
| | | | | 13/08/14 | NT | - | NT | 38.21 | 0 | PNC | Negative |
| **2** | AML | Positive | Probable | 07/02/14 | 2.184 | 0.82 | Positive | 32.1 | 31.69 | POS | *Positive* |
| **3** | ALL | Not requested | NEF | 17/02/14 | 3.744 | 1.60 | Positive | 44.26 | 0 | NEG | Negative |
| **4** | Wiskott-Aldrich Syndrome | No evidence of fungal disease | NEF | 16/10/14 | 0.20 | - | Negative | 0 | 0 | NEG | Negative |
| | | | | 22/01/15 | 0.853 | 0.675 | Positive | 0 | 0 | NEG | Negative |
| | | | | 29/01/15 | 0.86 | 1.271 | Positive | 0 | 0 | NEG | Negative |
| | | | | 26/01/15 | 1.01 | 0.646 | Positive | 0 | 0 | NEG | Negative |

GMI = galactomannan index, CT positive threshold for 28Asp PCR = 41, AML = acute myeloid leukemia, ALL = acute lymphoblastic leukemia,

NEF = No evidence of fungal disease.

### 9.3.8 Clinical Performance of *Aspergillus* PCR

Performance of the 28Asp and the Fungiplex PCR was measured against the clinical definition of IFI as the gold standard. The first analysis only considered probable patients as those with a diagnosis of IFI (table 9.8). The second analysis included possible and probable patients as those with a diagnosis of IFI (table 9.9).

Performance of the 28Asp PCR assay was assessed using two definitions of a positive result. A single positive PCR reaction (out of two replicates) was termed positive non-confirmed (PNC) and when the two replicate PCR reactions were positive the result was termed POS. Performance of the 28Asp PCR was undertaken to include and exclude PNC results. The Fungiplex PCR was performed as a single reaction therefore; a single positive was considered as positive.

### 9.3.9 PCR performance for patients with a diagnosis of probable IFI

#### 9.3.9.1 POS/PNC 28Asp PCR performance

28Asp PCR demonstrated a sensitivity of 100% (2/2, CI: 34.2, 100), specificity of 69.2% (56/81 CI: 58.4, 78.1), PPV 7.4 (2/27, CI: 2, 23.4) and NPV of 100% (56/56, CI: 93.6, 100) for the diagnosis of IA in probable patients when considering POS and PNC results as positive. The overall diagnostic accuracy was 69.9% (58/83, CI: 59.3 78.7) and the positive likelihood ratio was 3.2 (CI: 3-3.5).

#### 9.3.9.2 POS 28Asp PCR performance

28Asp PCR demonstrated a sensitivity of 100% (2/2, CI: 34.2, 100), specificity of 92.6% (76/81 CI: 86.4, 97.3), PPV 28.6% (2/7, CI: 8.2, 64.1) and NPV of 100% (76/76, CI: 95.2 100) for the diagnosis of IA in probable patients when considering only POS results as positive. The overall diagnostic accuracy was 94% (77/83, CI: 86.7, 97.4) and the positive likelihood ratio was 16.2 (CI: 11-24).

#### 9.3.9.3 Fungiplex PCR

Fungiplex PCR demonstrated a sensitivity of 100% (2/2, CI: 34.2, 100), specificity of 61.7% (50/81, CI 50.8, 71.6), PPV 6.1% (2/33, CI: 1.7, 19.6) and NPV of 100% (50/50, CI: 92.9, 100) for the diagnosis of IA in probable patients. The overall diagnostic accuracy was 62.7% (52/83, CI: 51.9, 72.2) and the positive likelihood ratio was 2.6 (CI: 2.4, 2.8).

**Table 9.8. Overall performance of the Fungiplex PCR-SERS and 28Asp qPCR for the diagnosis of invasive Aspergillosis in 83 patients with Probable IA.**

| | 28Asp PNC$/POS* | | 28Asp POS | | Fungiplex | |
|---|---|---|---|---|---|---|
| | *%* | **95% CI** | *%* | **95% CI** | *%* | **95% CI** |
| Sensitivity | 100 | 34.2, 100 | 100 | 34.2, 100 | 100 | 34,2, 100 |
| Specificity | 69.2 | 58.4, 78.1 | 93.8 | 86.4, 97.3 | 61.7 | 50.8, 71.6 |
| NPV | 100 | 93.6, 100 | 100 | 95.2, 100 | 100 | 92.9, 100 |
| PPV | 7.4 | 2, 23.4 | 28.6 | 8.2, 64.1 | 6.1 | 1.7, 19.6 |
| Diagnostic accuracy | 69.9 | 59.3, 78.7 | 94 | 86.7, 97.4 | 62.7 | 51.9, 72.2 |
| LHR+ | 3.24 | 3 – 3.5 | 16.2 | 11, 24 | 2.6 | 2.4, 2.8 |
| LHR- | 0 | 0.0 - ? | 0 | 0.0 – ? | 0.0 | 0.0 – ? |

*POS = two positive PCR replicates $PNC = Positive not confirmed with one positive reaction.

### 9.3.10 PCR performance for patients with a diagnosis of probable or possible IFI

#### 9.3.10.1 POS/PNC 28Asp PCR performance

28Asp PCR demonstrated a sensitivity of 50% (5/10, CI: 23.7, 76.3), specificity of 69.9% (51/73 CI: 58.6, 79.2), PPV 18.5% (5/27, CI: 8.1, 36.7) and NPV of 91.2% (51/56, CI: 80.7, 96.1) for the diagnosis of IA in probable/possible patients when considering POS and PNC results as positive. The overall diagnostic accuracy was 67.5% (56/83 CI: 56.8, 76.6) and the positive likelihood ratio was 1.7 (CI: 1, 2.7). The negative likelihood ratio was 0.7 (CI: 0.5, 1.1).

#### 9.3.10.2 POS 28Asp PCR performance

28Asp PCR demonstrated a sensitivity of 20% (2/10, CI: 5.7, 51), specificity of 93.2% (68/73 CI: 85, 97), PPV 28.6% (2/7 CI: 8.2, 64.1) and NPV of 89.5% (68/76, CI: 80.5, 94.6) for the diagnosis of IA in probable/possible patients when considering only POS results as positive. The overall diagnostic accuracy was 84.3% (70/83, CI: 75, 90.6) and the positive likelihood ratio was 2.9 (CI: 0.03-217.8). The negative likelihood ratio was 0.85 (CI: 0.7, 1.1).

### 9.3.10.3 Fungiplex PCR

Fungiplex PCR demonstrated a sensitivity of 50% (5/10 CI: 23.6, 76.3), specificity of 61.6% (45/73 CI: 50.2, 72), PPV 15.5 (5/27 CI: 6.7, 30.9) and NPV of 90% (51/56, CI: 78.6, 95.7) for the diagnosis of IA in probable/possible patients when considering only POS results as positive. The overall diagnostic accuracy was 60.2% (56/83 CI: 49.5, 70.1) and the positive likelihood ratio was 1.3 (CI: 0.8-2.1). The negative likelihood ratio was 0.8 (CI: 0.5 1.2).

**Table 9.9 Overall performance of the Fungiplex PCR-SERS and 28Asp qPCR for the diagnosis of invasive aspergillosis in patients with Probable or Possible IA.**

| | **28Asp PNC$^{\$}$/POS*** | | **28Asp POS** | | **Fungiplex** | |
|---|---|---|---|---|---|---|
| | ***%*** | **95% CI** | ***%*** | **95% CI** | ***%*** | **95% CI** |
| Sensitivity | 50 | 23.7, 76.3 | 20 | 5.7, 51.0 | 50 | 23.6, 76.3 |
| Specificity | 69.9 | 58.6, 79.2 | 93.2 | 85, 97 | 61.6 | 50.2, 72 |
| NPV | 91.2 | 80.7, 96.1 | 89.5 | 80.5, 94.6 | 90 | 78.6, 95.7 |
| PPV | 18.5 | 8.1, 36.7 | 28.6 | 8.2, 64.1 | 15.5 | 6.7, 30.9 |
| Diagnostic accuracy | 67.5 | 56.8, 76.6 | 84.3 | 75, 90.6 | 60.2 | 49.5, 70.1 |
| LHR+ | 1.7 | 1, 2.7 | 2.9 | 0.03, 217.8 | 1.3 | 0.8, 2.1 |
| LHR- | 0.7 | 0.5, 1.1 | 0.85 | 0.7, 1.1 | 0.8 | 0.5, 1.2 |

*POS = two positive PCR replicates $PNC = Positive not confirmed with one positive reaction.

### 9.3.11 Comparison of PCR positivity by Fungiplex PCR-SERS and 28Asp qPCR

In total 14.3% (49/342) of samples were positive by 28Asp PCR and (43/342) as detailed in table 9.10. Overall agreement between the two assays was seen for 79.9% of the samples. Out of 85 samples positive by either assay only 8.2% (7/85) were positive by both assays. This agreement is considered to be poor between the two assays.

| Table 9.10 Comparison of Fungiplex and qPCR results per sample. | | |
|---|---|---|
| | **qPCR Positive (+ve)** | **qPCR Negative (-ve)** |
| Fungiplex Positive (+ve) | 6 | 37 |
| Fungiplex Negative (-) | 32 | 268 |
| Agreement | 79.9% | - |
| Kappa | 0.035 | (-0.08-0.149) |

### 9.3.12 Performance of combined biomarker testing; Fungiplex PCR-SERS and 28Asp *q*PCR alongside GM EIA in patients with Probable IA

The performance of combined biomarker testing for the detection of IA in patients with probable disease is detailed in table 9.11. When combining a positive PCR result with either Fungiplex PCR-SERS or 28Asp *q*PCR with a positive GM EIA result for probable patients the sensitivity was 100% (34.2, 100), specificity was 100% (95.5, 100), PPV 100% (34.2, 100) and NPV 100% (95.5, 100).

| Table 9.11 Overall performances of the Fungiplex PCR-SERS and 28Asp qPCR assays in combination with Galactomannan EIA for the diagnosis of IA in probable and NEF patients. | | | | |
|---|---|---|---|---|
| | **Fungiplex PCR-SERS + GM** | | **28ASP PNC$^{\$}$/POS* + GM** | |
| | % | 95% CI | % | 95% CI |
| Sensitivity | 100 | 34.2, 100 | 100 | 34.2, 100 |
| Specificity | 100 | 95.47, 100 | 100 | 95.47, 100 |
| NPV | 100 | 95.47, 100 | 100 | 95.47, 100 |
| PPV | 100 | 34.2, 100 | 100 | 34.2, 10O |
| Diagnostic accuracy | 100 | 95.6, 100 | 100 | 95.58, 100 |

### 9.3.13 ROC analysis of $C_T$ values from the 28Asp PCR

Receiver operator characteristic (ROC) curves were plotted for four scenarios, POS PCR and probable disease figure 9.5 a), POS or PNC PCR with probable disease figure 9.5 b), POS PCR with possible/probable disease figure 9.5 c) and POS/PNC PCR with probable or possible disease classifications figure 9.5 d). The area under the curve was poor at 0.65 and 0.70 for POS 28Asp PCR and probable disease and POS or PNC 28Asp PCR with probable disease respectively. For the remaining two analyses the area under the curve of 0.55 and 0.57 indicated 'failed' performance of PCR in the POS 28Assp PCR with possible/probable disease and 28Asp POS/PNC PCR with probable or possible disease. Table 9.12 demonstrates the performance parameters of the 28Asp PCR in the four categories at the analytically determined LOD of the PCR, a $C_T$ of 41 cycles. Again this analysis supports the use of duplicate PCR reactions (POS) to be a true positive result for the diagnosis of IA with a sensitivity of 32% (CI: 15, 54), specificity of 97% (CI: 94, 98).

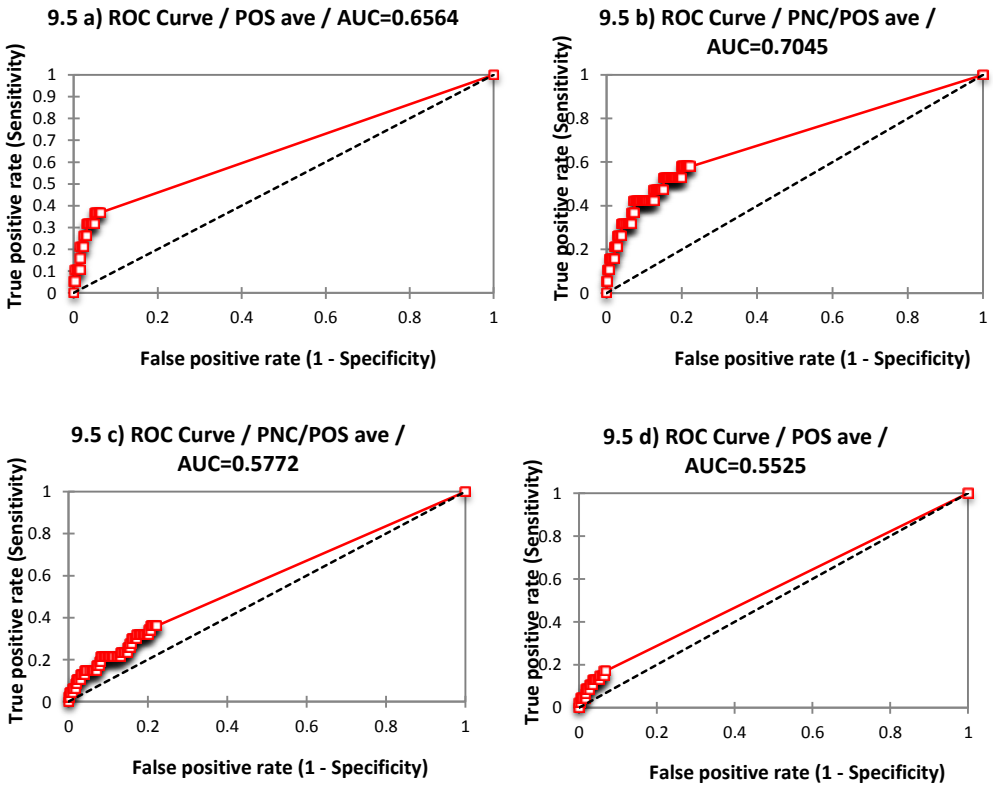



Figure 9.5 ROC curve analysis for 28Asp PCR for a) POS PCR (using an average CT) and probable disease, b) POS (using an average CT) /PNC PCR with probable disease c) POS PCR (using an average CT) and probable/possible disease and d) POS (using an average CT) /PNC PCR and probable/possible disease.

**Table 9.12 Overall performance of the 28Asp PCR at the positive $C_T$ threshold of 41 for PCR classifications against disease classification.**

| PCR | EORTC | Sens | % CI | | Spec | % CI | | PPV | NPV | LR+ | LR- | TP | TN | FP | FN | Acc |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| POC | Probable | 0.32 | 0.15 | 0.54 | 0.97 | 0.94 | 0.98 | 0.35 | 0.96 | 9.3 | 0.7 | 6 | 313 | 11 | 13 | 0.93 |
| POS/PNC | Probable | 0.42 | 0.23 | 0.64 | 0.92 | 0.88 | 0.94 | 0.23 | 0.96 | 5.2 | 0.62 | 8 | 298 | 26 | 11 | 0.89 |
| POS | Probable/possible | 0.13 | 0.06 | 0.27 | 0.96 | 0.93 | 0.98 | 0.35 | 0.87 | 3.4 | 0.90 | 6 | 285 | 11 | 41 | 0.84 |
| POS/PNC | Probable/possible | 0.21 | 0.12 | 0.35 | 0.92 | 0.88 | 0.95 | 0.29 | 0.88 | 2.6 | 0.86 | 10 | 272 | 24 | 37 | 0.82 |

Acc = accuracy, POS = two positive reactions, PNC = positive non-confirmed with one positive reaction

## 9.4 Discussion

This study has demonstrated the challenges of utilising PCR for the diagnosis of invasive aspergillosis in the haemato-oncology setting. Both PCR assays demonstrated the ability to exclude disease with NPV of 100% (56/56, 95% CI: 95.2, 100) for the 28Asp PCR (POS only) and 100% (50/50, 95% CI: 92.9, 100) for the Fungiplex PCR. Both assays detected *Aspergillus* sp in patients with probable disease, with a sensitivity of 100% (2/2, 95% CI: 34.2, 100). However, *Aspergillus* DNA was also detected from serum samples of patients without disease, and both assays demonstrated very low PPV, 28.6% (2/7, 95% CI: 8.2, 64.1) for the 28Asp assay (accepting POS only) and 6.1% (2/33, 95% CI: 1.7, 19.6) for the Fungiplex assay (when accepting only patient with probable disease). Specificity was improved for the 28Asp assay 93.8% (56/81 95% CI: 86.4, 97.3) by accepting duplicate PCR reactions as a true positive (POS) versus 61.7% (50/81 95% CI: 50.8, 71.6) for the Fungiplex assay that could only be performed as a single reaction. This study has demonstrated that *Aspergillus* PCR can be used to rule out Invasive aspergillosis but cannot be used to accurately detect disease as a stand-alone test. When combined with a second biomarker, in this case GM-EIA, PCR shows better statistical significance for diagnosing invasive aspergillosis infection.

Both PCR assays show promise for the accurate inclusion and exclusion of invasive aspergillosis when used in combination with a second biomarker such as GM-EIA. In this study 100% (83/83 95% CI: 95.6, 100) diagnostic accuracy for the detection of invasive aspergillosis, during the ‘at risk’ neutropenia window was demonstrated by the 28Adp and the Fungiplex PCR assays. These findings are supported by several other studies that also demonstrated increased accuracy of diagnosis when combining biomarkers along with clinical findings on CT (Rogers *et al.*, 2013; Johnson *et al.*, 2015; Paholcsek *et al.*, 2015; Eigl *et al.*, 2016). This discussion aims to consider the analytical performance of *Aspergillus* PCR and highlight the factors impacting on the clinical performance that contribute to lack of accuracy of PCR as a standalone test in the diagnosis of IA.

### 9.4.1 Analytical performance of *Aspergillus* PCR

It is important to implement a highly sensitive PCR strategy for the detection of *Aspergillus* DNA from serum. Free circulating DNA is known to be present in low quantities during the disease process (J Loeffler *et al.*, 2000; Challier *et al.*, 2004). In

2002 Loeffler and colleagues demonstrated through analysis of serum from mice models that the detection of *Aspergillus* in mice known to have invasive pulmonary disease was only 25%, and that the fungal load in blood was relatively low ($10^1$ – $10^2$ cfu/mL), in blood despite the mice having significant pulmonary infection (Loeffler *et al.*, 2002). It was also demonstrated that the quantities of DNA detected by PCR in the murine model of invasive aspergillosis were proportional to the amount of fungal biomass present in the mouse lung (Morton *et al.*, 2010), suggesting that in early infection the burden of DNA in blood would be low and corresponding to low burden in the lung. The observation made in the mouse model studies have correlated with observations made when testing blood samples from patients also known to have invasive aspergillosis (Oliver Morton *et al.*, 2011). As the diagnostic aim is to apply PCR to detect the early stages of infection it is essential to employ a highly sensitive PCR to detect these low levels of circulating DNA.

The first step of this study was to demonstrate that both PCR assays evaluated were sensitive enough to detect low quantities of DNA from serum. Using control plasmid for the 28Asp assay sensitivity down to 20 plasmid copies per reaction equivalent to 1 genome copy per reaction was demonstrated. In a previous study the LOD of Fungiplex was reportedly 20 plasmid copies per reaction with 95% reproducibility (White *et al.*, 2014). However, for this study plasmid was not available to assess the Fungiplex sensitivity. The comparison of sensitivity of the two PCR assays evaluated was achieved through dilutions of genomic DNA, which were analysed. Absolute reproducibility was demonstrated at concentrations equivalent to 6.5 (CI: 2.8, 14.4) plasmid copies copies/µL for both assays, equivalent to between 1 and 7 genome copies per reaction. The detection of *Aspergillus* DNA was possible at the lowest concentration of genomic DNA equivalent to between 0.3 and 3 genome copies per µL, but was not 100% reproducible at this dilution for both assays, most likely reflecting the variability in the lowest genomic DNA concentration. These observations are supported by work published by the EAPCRI working party which demonstrated 97% reproducibility at a concentration of 10 genomes per mL of serum, equivalent to 2 genome copies per reaction (White *et al.*, 2011). As comparable sensitivity was observed across both assays it can be concluded that if any differences in performance were observed it is unlikely that this would be due to differences in sensitivity. A limitation of this study was not to investigate dilutions between the medium and low concentrations therefore, one can only state that the LOD lies between 1-7 genome copies per reaction. As both assays demonstrate the sensitivity required follow-up investigations were required to assess the impact of DNA extraction and sample input

volume to ensure the DNA extraction process did not negatively impact on the sensitivity of the workflow. The extraction methods in this study followed the guidance published by the EAPCRI working party.

### 9.4.2 EAPCRI compliant DNA extraction

Over the past decade the EAPCRI working party have worked to develop guidelines that can be used across clinical centres to standardize *Aspergillus* PCR (White *et al.*, 2010, 2011, 2012). Work conducted to date has identified the critical factors influencing the performance of *Aspergillus* PCR through the distribution of blinded quality control (QC) panels to international centres. The results from the working party identified that DNA extraction was *the* critical component of the PCR process that affected the performance of *Aspergillus* PCR. The recommendations made by EAPCRI are to use a commercial extraction method, preferably automated extracting from a minimum of 500µL of sample and elute into less than 100µL of elution buffer (White *et al.*, 2010, 2011, 2012). To ensure optimal performance of both PCRs being investigated in this study we chose to investigate the impact of extracting from two sample volumes, 550µL and 1mL, whilst maintaining the eluate to a 50µL volume on the automated extraction platform Diasorin IXT.

### 9.4.3 Impact of sample volume DNA extraction

By spiking serum with the genomic DNA dilutions previously characterized through direct PCR analysis it was possible to demonstrate the impact of the extraction process and input sample volume on recovery of DNA. No loss in sensitivity was observed when analysing extracts from 1mL of serum for high concentrated samples, whereas $C_T$ values were significantly higher when extracting from 550µL of serum, indicating a loss in sensitivity. A significant loss in sensitivity was observed when extracting DNA from 1mL of serum at the medium concentration but 100% reproducibility was still maintained, whereas from the 550µL serum extract the detection of *Aspergillus* was no longer 100% reproducible. It is expected that some loss in DNA will occur through extraction; this observation was demonstrated by White *et al* whereby DNA that was extracted and quantified revealed losses of at least $10^2$ copies during manual extraction. Minimising the loss of DNA during extraction through the use of novel automated extraction technology has been recommended by leading groups investigating the performance of *Aspergillus* PCR (White, Linton, *et al.*, 2006).

This study aimed to minimize the impact by utilising automated extraction with paramagnetic bead-based capture of DNA. The Diasorin IXT (Nordiag Arrow) has been shown to be highly efficient nucleic acid platform when extracting from blood cultures, stool and spiked blood samples (Laakso *et al.*, 2011; Laakso and Mäki, 2013; Kalina *et al.*, 2014; Forsell *et al.*, 2015). When extracting DNA from whole cell bacteria spiked into blood the method was shown to be sensitive at 11cfu/mL for *Escherichia coli*. To date there are no comparative studies published using this exact method of extraction for *Aspergillus* DNA from serum but other similar platforms utilising paramagnetic bead-based DNA capture have been shown to be superior to column based and manual extraction techniques through increased yield of nucleic acid from paraffin embedded tissue (Ribeiro-Silva, Zhang and Jeffrey, 2007). When extracting 4-5 *A. fumigatus* genome copies from 1mL of serum ultimately equating to 1-2 genomes copies input per reaction this study demonstrated that 100% reproducibility through both PCR assays. It can be concluded that the Diasorin IXT extraction platform used in conjunction with both PCR assays combined to produce a laboratory workflow met the required performance characteristics expected of a diagnostic assay in the clinical setting of invasive aspergillosis.

### 9.4.4 Clinical performance of PCR

The gold standard for comparison of PCR performance in this study was the classification of patients according to 2008 EORTC/MSG criteria (De Pauw *et al*. 2008) into categories of proven, probable, possible disease or control cases. The primary analysis was assessing the performance of PCR for the diagnosis of IA in patients with proven/probable IA. Patients classified as having possible disease were included alongside those without evidence of disease in the control group. This is the approach recommended by the Cochrane group (Leeflang *et al.*, 2013) as possible IA should not be considered as positive for treatment purposes. In clinical practice many patients are classified as possible cases of IA, therefore a secondary analysis was performed to assess PCR performance for the diagnosis of patients classified as probable and possible cases of IA versus control patients.

### 9.4.5 Prevalence of Invasive Aspergillosis according to EORTC MSG criteria

In this study the prevalence of probable and probable/possible invasive aspergillosis was 2.4% (2/83) and 12% (10/83) respectively. We did not experience a case of

proven invasive aspergillosis. In a recent meta-analysis evaluating the performance of *Aspergillus* PCR the mean incidence of proven/probable diseases across the 13 published studies was 22.5% (range 6.5%-66.7%) (Arvanitis, Anagnostou and Mylonakis, 2015). The mean number of patients included in the respective studies was 118 (range 25-549) with 8 studies having less than 100 patients. The sample size of these studies is not too dissimilar from this study therefore; one can conclude that in this study the prevalence of invasive aspergillosis is low in comparison to other published work. The use of mould active antifungals as prophylaxis or empirically in patients at high risk of invasive fungal disease in our centre is most likely the cause of this observation, resulting most likely resulted in the low prevalence of disease. This is supported by a study by Cordonnier *et al* who demonstrated that patients receiving empirical antifungal therapy the prevalence of disease was lower at 2.7% versus 9.1% in the pre-emptive study arm who did not receive mould active treatment empirically (Cordonnier *et al.*, 2009). The prevalence of disease is important when assessing the performance of diagnostic assays, in low prevalence disease settings the specificity and PPV of diagnostic tests is reduced (Leeflang *et al.*, 2013). The performance characteristics of a diagnostic test determine how the test is utilized in the management of patients. If the test is highly specific with a high PPV then it may be used to confirm the presence of disease, whereas tests with high NPV and sensitivity can be used, when negative, to rule out disease (diagnostic tests 2: predictive values bland). In this study *Aspergillus* PCR demonstrated the latter attribute.

### 9.4.6 Negative predictive value of *Aspergillus* PCR

This study has demonstrated that the strength of *Aspergillus* PCR is in its ability to rule out disease in patients at risk of invasive aspergillosis with NPV of 100% (76/76, CI: 95.2 100) for the 28Asp PCR (POS) and 100% (50/50, CI: 92.9, 100) for the Fungiplex PCR when considering patients with probable disease only. High NPV for the diagnosis of proven/probable IA is a finding replicated in many studies investigating *Aspergillus* PCR for the diagnosis of IA (Mengoli *et al.*, 2009; Arvanitis, Anagnostou and Mylonakis, 2015). When considering patients with probable/possible disease the NPV was reduced to approximately 93.2% in our study. A definition of possible IA does not rely on the inclusion of a fungal biomarker leading to a less stringently defined cohort of patents, in which patients without disease will almost definitely be included as cases. It is generally accepted that when using PCR assays in clinical practice disease cannot be conclusively ruled out in a small proportion of patients. However, the NPV of PCR

remains the leading attribute suggesting PCR as an ideal *Candida*te as a diagnostic test to drive pre-emptive strategies when prescribing antifungal agents in clinical practice.

The use of PCR to rule out invasive aspergillosis or to withhold unnecessary use antifungal agents is referred to as a targeted pre-emptive approach to antifungal therapy. A clear definition of a 'pre-emptive antifungal strategy' has not been established in national or international guidelines, but commonly the aim is to restrict the use of empirical antifungal therapy to those who have an indication of early stage antifungal disease. This would reduce the overall use of antifungals and their associated costs. Furthermore it would lessen the attributed mortality from toxicity. Antifungal therapy may then be administered when either radiologically or a biomarker positive (PCR or GM EIA) results are suggestive (Maertens *et al.*, 2006; Cordonnier *et al.*, 2009; Barnes *et al.*, 2013; Knitsch *et al.*, 2015). This approach is increasingly necessary as the use of antifungals in healthcare is disproportionate to the incidence of invasive fungal disease. It is estimated that over 50% of antifungals are inappropriately administered (J Loeffler *et al.*, 2000; Sutepvarnon *et al.*, 2008; Nivoix *et al.*, 2012). Antifungal agents are toxic to certain patients as many of the molecular targets for antifungal drugs are eukaryotic molecules synonymous with those within the human body, in particular those resulting in hepatic and renal toxicity. Moreover, antifungal agents can be expensive, with the annual UK antifungal spend exceeding £112 million alone in 2013 (PHE, 2014). There are also only four main classes of antifungal agents used empirically and with antifungal resistance increasing and multi-drug resistant (MDR) fungi such as *C. auris*, are emerging globally under the selective pressure of antifungal overuse (Chowdhary *et al.*, 2014; Morales-López *et al.*, 2017). Selective use of antifungal agents and antifungal stewardship is now a healthcare directive from the PHE with the increased risk of MDR yeast species such as *C. auris* (PHE, 2016).

One limitation of this study was that data relating to antifungal administration to patients was not collected so for our patient cohort not allowing estimates are made as to how PCR may have affected antifungal use in a targeted approach. However, our management strategy for haemato-oncology patients is to use Itraconazole prophylaxis (or liposomal Amphotericin B when Itraconazole is contraindicated) during their period of neutropenia to protect against IA. In this study using the 28Asp PCR or the Fungiplex PCR with two positive reactions 62.7% (52/83) patients tested negative for *Aspergillus sp* and according to EORTC/MSG criteria were classified as control patients unlikely of ever having Invasive Aspergillosis. Prophylaxis could have been withheld in a significant proportion of these patients based on biomarker use. This is

supported by findings in published studies utilising a pre-emptive approach to antifungal therapy (Maertens *et al.*, 2005; Girmenia *et al.*, 2010; Tan *et al.*, 2011; Barnes *et al.*, 2013).

In 2013, Barnes and colleagues published their findings when assessing a pre-emptive pathway of twice weekly antigen and PCR screening for the management of invasive fungal disease (Barnes *et al.*, 2013). In this study 549 high-risk haematology and stem cell transplantation patients were enrolled over a 5-year period. Both GM-EIA and PCR showed optimal performance as screening tests when used in combination with high sensitivity (98%) and negative predictive value (99.6%). Using a pre-emptive strategy a £100,000 decrease in antifungal spends over a six month period was observed (Barnes *et al.*, 2013). These savings were approximately double the cost of implementing the biomarker screening (Barnes *et al.*, 2013). Using a pre-emptive approach through a diagnostic driven strategy also resulted in a significant decrease (p=0·002) in antifungal use in a randomized control trial (Morrissey *et al.*, 2013).

Caution must be taken when implementing any diagnostic driven strategy, incorrect implementation may result in overuse of antifungal agents. In 2009 Herbert and colleagues utilized a pan fungal PCR to drive pre-emptive use of antifungal agents compared with a fever based driven strategy in the empirical group (Hebart *et al.*, 2009). An increase in antifungal usage was observed in the pre-emptive arm as the investigators chose to administer antifungals after 5 days of fever despite obtaining negative PCR results, disregarding the NPV of fungal PCR (Hebart *et al.*, 2009). It is also important to acknowledge that if empirical treatment is withheld in patients at risk of invasive aspergillosis and a pre-emptive approach is used to drive antifungal therapy an increase in the prevalence of aspergillosis may be observed. Whereas work performed by Cordonier *and colleagues observed* significantly increased prevalence ($p<0.02$) of aspergillosis of 9.1% compared with 2.7% in the preemptive arm, but also observed a reduction in the use of antifungals in the preemptive arm (Cordonnier *et al.*, 2009). It may be the case that removing empirical therapy enables the performance of the diagnostic assays to improve. Antifungal treatment has been shown to affect the performance of PCR in early stages of disease, reducing sensitivity of detection in animal models (McCulloch *et al.*, 2012) and in patients at risk of invasive aspergillosis (Marr *et al.*, 2004). The reduction in biomarker availability when using antifungal prophylaxis with a background of low disease prevalence requires the use of a highly sensitive PCR assay. In this setting the effects of false positivity in the diagnostic workflow are enhanced, as was observed in this study.

### 9.4.7 Positive predictive value and specificity of *Aspergillus* PCR

The quantity of fungal nucleic acid in serum is low (J Loeffler *et al.*, 2000; Challier *et al.*, 2004) and is most likely very close to the limit of PCR detection (LOD) (Springer *et al.*, 2013). Previous studies have recommended that a single positive reaction should be accepted as positive, even when performing PCR in duplicate reactions (Millon *et al.*, 2005; Springer *et al.*, 2012). In this study, when accepting a single positive PCR reaction as a true positive, the performance of the two PCR assays was comparable with a very low PPV of 7.4% (2/27, CI: 2, 23.4) for the 28Asp PCR and 6.1% (2/33, CI: 1.7, 19.6) for the Fungiplex assay. Specificity was also low at 69.2% and 61.7% for the 28Asp and Fungiplex assays respectively. If the positive acceptance criteria were restricted to only accept a true positive result as a sample that is reproducibly positive (two positive PCR reactions) performance of the 28Asp PCR was improved to a PPV and specificity of 28.6% (2/7, CI: 8.2, 64.1) and 92.6% (76/81 CI: 86.4, 97.3). This performance is comparable to that of the meta-analysis published by Arvantis and colleagues (Arvanitis, Anagnostou and Mylonakis, 2015). This study has demonstrated that applying PCR in a very low disease prevalence setting, with only two cases of probable disease more stringent criteria are required to obtain the performance expected of PCR as a diagnostic assay. The same analysis could not be performed for the Fungiplex as the study was limited by the cost at £45 per single reaction. It is possible that the performance of the Fungiplex assay would have also been improved by performing PCR in duplicates. The performance of the Fungiplex PCR was previously shown to be 85.7% and specificity of 87.5% with single reactions which increased to a specificity of 97.5% when testing in duplicate (White *et al.*, 2014). Performance of PCR can also be enhanced by combination with a second biomarker for the detection of *Aspergillus*.

The combination of Galactomannan and PCR for the diagnosis of IA in probable patients was shown to be 100% accurate in this study, although was limited by only two patients being classified as having probable IA. Galactomannan and PCR were only positive in combination for patients with radiological evidence of disease. The impact is slightly biased as a probable case of IA is defined as being a patient with a positive GM EIA, but no patients were positive by both biomarkers in the absence of radiological evidence of disease. The combined biomarker approach has been shown to be successful in other studies. In 2008 Botterel and colleagues demonstrated improved specificity for the diagnosis of IA when performing PCR on the first GM positive sample when screening liver transplant patient at risk of IA (Botterel *et al.*, 2008). Aguado *et al* demonstrated that earlier diagnosis could be achieved using PCR

and GM when compared with GM alone with a median time to diagnosis of 13 days versus 20 days respectively (Aguado *et al.*, 2015). Furthermore survival was statistically higher in the PCR-GM group in this study ($p<0.05$) (Aguado *et al.*, 2015). Combined use of GM and PCR could neutralize the effects of false positivity when using PCR as a stand-alone assay.

The risk is that a false positive result could lead to the administration of unnecessary antifungal therapy. In this study if a pre-emptive approach had been driven by one positive PCR reaction 32.5% (27/83) of patients would have been administered targeted antifungal therapy and 39.8% (33/83) if the Fungiplex assay had been utilized. This echoes the findings of the study by Hebart [discussed previously] who observed the use of antifungal agents in the pre-emptive fever plus PCR arm increase by 20.4% (Hebart *et al.*, 2009). The high rate of positive results when using *Aspergillus* PCR diagnostically is a concern and the environment in which the PCR is performed undoubtedly is a key factor. False positivity when utilising a highly sensitive PCR is almost unavoidable in this setting as *Aspergillus* is ubiquitous in the environment. As saprophytic fungi, *Aspergillus sp* are found in soil and detritus material and is freely cultured from the environment through an abundance of conidia dispersed though the aerodynamic structure of spores. As a consequence via both conidia but also free DNA in the environment PCR reaction processes can be readily contaminated. *A. fumigatus* has been found to contaminate extraction reagents in commercially available kits and blood vacutainers used to collect blood specimens (Rimek *et al.*, 1999; Harrison *et al.*, 2010; Perry, White and Barnes, 2014). A further reported source of false positivity is a lack of fungal specificity in the design of PCR assays which may result in the amplification human DNA, which has also been previously reported (White and Barnes, 2006; White,*et al.*, 2006). To reduce the impact of false positivity this study a cut off of 41 cycles was selected. The rationale for this threshold was that an input of 3 plasmid copies into the reaction resulted in a median CT of 40.8 that was equivalent to the LOD of our assay at evaluation. Any positivity beyond a cycle threshold of 41 most likely does not represent true amplification but late false amplification in the assay. One further complication experienced in this study which almost certainly contributed to the high rate of false positivity was in the design of the assays. The 28Asp PCR targets the gene encoding 28S ribosomal RNA (White *et al*, 2011). The Fungiplex PCR also targets this same region. When running these two workflows in parallel it became clear that the rate of false positively was higher than would have been expected.

The root cause of the increased false positivity was poor control of cross contamination in the environment in which the assays were being performed in, resulting in carry over

contamination. The Fungiplex workflow requires amplification tubes to be opened for the addition of the amplification product into the hybridization reaction. This action is a high risk process that should ideally be performed in a post-PCR environment, i.e. a room dedicated for the handling of amplicon manipulation, completely isolated from reagent preparation (pre-PCR) and other sample (DNA extraction) working environments. Furthermore, once this task has been performed the operator should not enter a clean environment until the next day once clothing has been changed as amplicons can be carried into clean environments (this process is similar to other high risk work flows in molecular pathology in areas such as pre-natal diagnostics). This study was conducted in a shared university research department where the control of amplicon spread was challenging due to lack of segregated molecular infrastructure. The correct molecular infrastructure in the clinical setting is crucial to establishing a robust service. The lack of a second open tube amplicon manipulation post-PCR room controlling for both movement of people and sample through the PCR workflow led to amplicon contamination being detected in the clean room. When testing the amplicon of the Fungiplex assay in the 28Asp PCR it was realized that the same target region was being amplified as positive reactions were obtained.

### 9.4.8 Conclusions

In conclusion, this study has demonstrated the challenges faced when using *Aspergillus* PCR for the diagnosis of IA in the haemato-oncology setting. Prophylaxis impacts on the performance of an assay already challenged by a low prevalence of disease. Environmental false positivity is a significant risk that could contribute to the over use of antifungal agents and misdiagnosis of infection. The 28Asp assay demonstrated the most successful approach when used in combination with GM, for diagnosis of IA. The Fungiplex PCR did not demonstrate any increased performance when compared with the 28Asp assay, despite its use of SERS technology. The assay setup process presents a significant risk when establishing *Aspergillus* PCR as a diagnostic test with high risk of cross contamination during post-PCR amplicon manipulation phase, therefore limiting its practicality in the diagnostic setting. In summary *Aspergillus* PCR remains a controversial test for diagnosis of IA and a multicentre trial investigating a pre-emptive strategy is most likely required to definitively answer the question as to whether this could be an effective approach.

# Chapter 10 Conclusion and Future Developments

Our research has demonstrated that it is possible to improve the diagnosis of invasive fungal disease by utilising molecular methods in the clinical diagnostic laboratory. At the outset of this research our routine diagnostic workflows relied heavily on culture based investigation and the detection of antigen using the GM–EIA. The aim of this research was to replace or supplement the existing testing strategy with molecular based techniques that could improve diagnosis of invasive fungal infection. Figure 10.2 details what is now the outputs of this research and is new our workflow for diagnosis of invasive fungal disease within our clinical service. Whilst, in some instances, the assays utilised in the respective research did not conclusively improve the diagnosis of IFI as stand-alone tests, their use in testing strategies has aided in informing how molecular tests can be utilised appropriately. One molecular method that has met the criteria required to be a stand-alone assay for diagnosis is MALDI-TOF MS for the identification of fungi.

## 10.1 Improving the identification of fungi from culture

The introduction of MALDI-TOF MS for the identification of yeasts from clinical specimens has undoubtedly revolutionised our approach to diagnosing invasive candidiasis. One of the main advantages of MALDI-TOF MS is an increased specificity in identifying rare yeast species from infections. After the introduction of MALDI-TOF MS based diagnosis of invasive candidiasis we diagnosed a rare infection of *C. nivariensis* in a renal patient with persistent Candiduria (Gorton *et al.*, 2013). In immunocompetent patients *Candida* in the urine is often considered insignificant, but in a renal patient cohort the isolation of *Candida* from urine warrants further investigation as ascending or descending renal infection post-transplant can be a major cause of morbidity and mortality (Safdar. *et al.*, 2005). The history of this patient when using AuxaColor (BIORAD) testing up to this point had been clinically confusing, as *C. nivariensis* isolates had been identified as two closely related species *Candida glabrata* (3/5) and *Candida inconspicua* (2/5) (Gorton *et al.*, 2013). This had led to the isolation of these yeasts being considered insignificant for over a year after the patient's admission to hospital. Current guidelines from the British Society of Medial Mycology strongly recommend that all *Candida* isolates from urine in the transplant setting are fully identified (Schelenz *et al.*, 2009, 2015). Biochemical profiling is currently the primary method for yeast identification in most diagnostic laboratories. As a result,

cryptic *de novo* species such as *C. nivarensis* are not incorporated into databases for commercially available biochemical assays and clinically significant yeasts are misidentified (Posteraro *et al.*, 2015). *C. nivariensis* is now a recognised cause of vaginal thrush and resistant to fluconazole, therefore demonstrating the impact of MALDI-TOF MS in benefiting routine clinical infection in addition to IFI. To date, several other case reports have detailed the impact of MALDI-TOF MS in the identification of rare yeast infection. These include the isolation of *Kodamaea ohmeri* in a case of fungemia which was correctly identified by MALDI-TOF MS (Valenza *et al.*, 2006; Distasi *et al.*, 2015; Aznar-Marin *et al.*, 2016). This reinforces the fact that MALDI-TOF MS is equivalent to gold standard methods such as ribosomal sequenced based identification (Borman *et al.*, 2008, 2010).

Perhaps the most important event to have occurred in the last 5 years from a clinical mycology perspective is the global emergence of the multi drug resistant species *Candida auris* (Chowdhary *et al.*, 2014; Clancy and Nguyen, 2017). In the UK, the emergence of *C. auris* is linked to carriage of the yeast into the UK through travel, as strains have been shown to have wide geographic origins (Borman, Szekely and Johnson, 2017). Intrinsically resistant to fluconazole, this species also has demonstrated high MICs to other antifungal agents such as amphotericin B and caspofungin (Arendrup and Patterson, 2017). Strains from India were reportedly fully resistant to all antifungal drug classes (Chowdhary *et al.*, 2014). *C. auris* now presents as a major infection control concern, as the opportunistic pathogen is able to colonise and cause invasive infection in vulnerable patients, most commonly in the ICU (Shetty *et al.*, 2016; Clancy and Nguyen, 2017). In a similar way to *Staphylococcus aureus* and MRSA, *C. auris* has resulted in large outbreaks and attributed deaths in healthcare systems globally. Biochemical methods, including CHROMagar and reaction test strips, are unable to distinguish this species from *C. haemulonii* or *C. duboshealulonii* (Kathuria *et al.*, 2015). MALDI-TOF MS is capable of identifying *C. auris* with a high degree of accuracy (Grenfell *et al.*, 2016). In the infection control setting, this technology now sits at the front line in the laboratory for the rapid identification of yeasts from cultures and has been essential in the control of clinical outbreaks. In centers where MALDI-TOF MS is not available, referral to recognised mycology reference services or implementation of molecular sequenced based identification techniques is the only other robust mechanism for identification. Both of these scenarios come with limitations in terms of both TAT and cost (Kordalewska *et al.*, 2017). Conclusively MALDI-TOF has strengthened the diagnostic laboratories ability to identify yeasts from clinical culture specimens.

### 10.2 Filamentous mould identification

The identification of filamentous moulds using MALDI-TOF MS in the clinical laboratory has also had success, with large multicentre efforts to establish a comprehensive databases that can be used with high degrees of accuracy (Lima, Santos and Vena, 2010; Del Chierico *et al.*, 2012; Kondori *et al.*, 2014; Ranque *et al.*, 2014). However, standardisation across processing methods used across centres has become an issue with deviation from manufacturer's recommended methods when establishing in-house databases (Lima, Santos and Vena, 2010; Del Chierico *et al.*, 2012; Kondori *et al.*, 2014; Ranque *et al.*, 2014). For our local clinical service we have decided to develop the existing Bruker filamentous mould database, in real time, using the manufacturer's recommended preparation methods. This maintains the validation criteria performed by the manufacturer and builds on the quality and breadth of the reference spectra. The implementation of MALDI-TOF MS alongside microscopic culture strengthens the laboratories ability to have confidence in identifications returned by less experienced scientists and if discordance is observed between the scientist and the MALDI-TOF MS identification the isolate can then be processed using molecular sequencing methods to allow for resolution of the species identification. In turn, this enables our service to develop the database by creating spectral profiles for unidentified or discrepant fungi using the ITS rRNA sequenced based identification as the gold standard.

Optimisation of the MALDI-TOF MS process could improve success. Normand and colleagues investigated a decision-base strategy against an in-house developed database and the commercially available filamentous mould dataset from Bruker. Across both databases they demonstrated that improved identification could be achieved by accepting that the highest score of four spots was taken into account with a 1.7 log (score) threshold (Normand *et al.*, 2013). Using the in house database 87.41% of isolates were identified compared to 35.15% using the Bruker database, with a positive predictive value (PPV) of 1 at the genus level for both databases as well as 0.89 PPV (in-house database) and 0.72 PPV (Bruker database) at the species level (Normand *et al.*, 2013). These efforts have contributed significantly to the knowledge base of filamentous mould diagnosis. In support of, but potentially superseding this, we have seen this year [2017] the release of a free open access MALDI-TOF MS database for filamentous fungi which aims to address the challenges discussed throughout this research.

https://biological-mass-spectrometry-identification.com/msi/

The database was the effort of a multicentre working party, the BCCM/IHEM collection in Brussels, whose aims were to build a database for fungi as robust as bacterial databases that currently exist in routine clinical diagnostics. Comprised of 11,851 spectra (938 fungal species and 246 fungal genera), the size of the database is significantly larger than commercial databases, which currently contain less than 150 reference spectra. The database has ISO:9001 certification and therefore is suitable for use in routine diagnostic laboratories and also supports commercial developments. A recent study evaluated the performance of the database against a collection of >600 isolates (Normand *et al.*, 2017). A total of 87.4% were correctly identified at the species level, while 5.21% were assigned to the correct genus but the incorrect species. Only 7.4% of the isolates were not identified as the defined threshold of 20 was not reached (Normand *et al.*, 2017). This approach is building on the success of the GenBank database for the identification of fungi through sequence-based analysis of unknown strains against an extensive database of characterized genomic sequences. However, like with all databases the strength of their accuracy is in the gold standard reference point used to create the database. The potential impact of the new web based databases will undoubtedly be far reaching with global implementation in the routine setting and will be one of the next objectives of our diagnostic laboratory and clinical service to introduce the new database into routine use.

### 10.3 Direct identification of yeasts from blood cultures

The limitation of MALDI-TOF for the identification of yeasts direct from blood culture specimens was the principal finding of our analysis using this technology, lacking specificity in its pan detection of proteins from clinical specimens. In a similar manner to the filamentous mould database, optimization for successful identification of yeasts direct from blood cultures using MALDI-TOF MS could be achieved by further investigating the process of sample preparation. In our study we utilized the Sepsityper kit. This was a limiting factor as the method was restricted in the processing steps that could be optimized. Ironically this was the most crucial element to the success of the technique. In a recent publication Jeddi and colleagues (2017) have demonstrated that an alternative method can significantly improve the success of MALDI-TOF MS for yeast species (Jeddi *et al.*, 2016). The method utilized by the group in brief involved processing 1.8mL of positive blood culture, which was first centrifuged for 13,000*g* for 2 minutes, the supernatant was removed, and the pellet was suspended in 1.8mL of sterile distilled water and centrifuged again at 13,000*g* for 2 minutes. The supernatant was removed and the pellet was suspended in 1.8mL of 0.1% SDS in sterile distilled

water and incubated for 10 minutes (Jeddi *et al.*, 2016). This contrasts with previous methods, as there is an initial centrifugation at high speed prior to processing the blood culture specimen through red cell lysis and wash steps, which was not investigated in our research. The difference in performance to the standard Sepsityper protocol was 98.9 v 66.6% success. As the *Quick*FISH assay is limited in its assay format this new method for yeast processing most likely requires some consideration when *Quick*FISH in our laboratory returns a result of no identification, figure 10.2.

To date, no further studies have been published using *Quick*FISH for the identification of yeasts direct from blood culture specimens. The test is fully implemented into our work streams for gram-positive bacteria and yeasts as a rapid method for identification. The results are being returned in real time along with the gram stain to clinicians and used for the antifungal decision-making process in patients with candidemia. The implementation of *Quick*FISH is a perfect example of a molecular test that was designed from the outset to complement the workflow of the clinical diagnostic laboratory without impact of staffing resource. Some of the most successful molecular diagnostic assays also follow this principle, including the GeneXpert (Cepheid, USA) range of NAAT assays in cartridge based format used widely in most *tuberculosis* laboratories globally (McNerney and Zumla, 2015). Expansion of the second generation *Quick*FISH assay will inevitably improve the assay and the next generation of the test will incorporate new species within the assay format, perhaps even a *Candida auris* probe.

The next frontier in the diagnosis of invasive candidiasis is undoubtedly the implementation of *Candida* PCR direct from blood alongside BDG testing for the detection of invasive Candidiasis. At the time of starting this research the BDG assay was confined to reference laboratory testing services. However, with the increasing use of the assay and the final implementation in our clinical laboratory, we are now starting to further investigate patients' who are positive by BDG. In these patients, for whom a clear diagnosis for IFI cannot be made, IC is the IFI for which we are lacking in a clear laboratory strategy and provides the next challenge diagnostically. The introduction of real-time PCR for *Candida* and the T2MR system (T2 Biosystems, MA, USA) in the future months will allow for a prospective evaluation of *Candida* PCR from blood alongside BDG for the diagnosis of invasive candidiasis (Mylonakis *et al.*, 2015; Pfaller, Wolk and Lowery, 2016). The T2MR has demonstrated excellent performance in studies to date reporting sensitivity and specificity of 91.1% and 92.3% respectively for the diagnosis of IC (Mylonakis *et al.*, 2015). Commercially available PCR kits are also

now available as real-time qualitative assays (Bruker Daltonics, Germany). The same principles and methods presented in the *Aspergillus* chapter will be applied to *Candida* PCR. This work is being undertaken as part of the efforts of the fungal PCR Initiative (FPCRI). With the use of PCR alongside the second biomarker BDG, which is not in itself a unique approach to invasive candidiasis is the approach we have implemented for the diagnosis of PCP in our clinical laboratory.

### 10.4 Diagnosis of PCP

GMS staining from BAL has been replaced by PCR as the front line test for the diagnosis of PCP. As demonstrated in this research, PCR can be limited in its specificity as colonisation of the lungs with *P. jirovecii* leads to false positive results. To resolve the lack of correlation between atypical clinical presentation for PCP and a low positive PCP PCR result we chose to adopt a dual biomarker testing strategy for improved clinical performance utilising the β-D-Glucan (BDG) assay (Fungitell, Cape Cod). The approach of dual biomarker testing is steadily increasing in adoption throughout centers using PCP PCR (Borstnar *et al.*, 2013; Wood *et al.*, 2013; White, Backx and Barnes, 2017).

In 2012 Matsumura and colleagues reported the first study to investigate the utilisation of dual biomarkers in patients being investigated for PCP (Matsumura *et al.*, 2012). A cut off of 1300 copies/mL of a single copy PCR targeting the dihydrophate synthase gene was established to discriminate between definite PCP with a specificity of 80.0%. A threshold of 340 copies/mL was used to discriminate between probable PCP and colonization (Matsumura *et al.*, 2012). Matsumura and colleagues measured BDG in the same patients and reported significantly higher BDG levels in the definite group (median 39.5pg/mL, range 15.7–352.7pg/mL) than in the colonized group (median 4.3, range 4.3–61.5, $p < 0.001$) (Matsumura *et al.*, 2012). In a similar investigation Damiani and colleagues demonstrated PCR had a PPV of 100% when the *P. jirovecii* DNA copy numbers were greater than $2 \times 10^4$ copies/μL (Damiani *et al.*, 2011). The negative predictive value of the assay was 100% when the *P. jirovecii* DNA copy numbers were less than $1.6 \times 10^3$ copies/μL (Damiani *et al.*, 2011). In figure 10.1 taken from Matsumara *et al* 2012 they correlated the BDG pg/mL results with the PCR copy numbers for each patient tested in the study. The authors demonstrated a clear relationship between low positive PCR results and positive BDG results (>100pg/mL) and suspected PCP infection. Where PCP infection was not suspected but the PCP reported a low positive result the BDG test was negative (<100pg/mL).

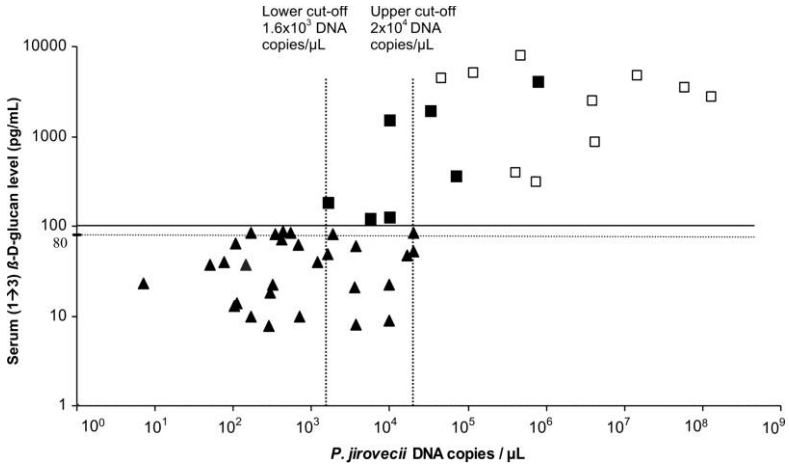



Figure 10.1 Correlation of β- D-glucan (pg/mL) and PCP *q*PCR (copies per µL) for the diagnosis of PCP in cases and controls (Damiani *et al.*, 2011).

Two further studies have since demonstrated that BDG levels are significantly different patients across the different classifications of disease made by incorporating PCR in the decision-making processes. Tasaka and colleagues reported β-D-glucan in the negative, colonization, probable PCP, and definite PCP subgroups as 20.2 ± 6.3, 48.8 ± 15.9, 89.9 ± 20.2, 224.9 ± 25.9 pg/mL (Tasaka, 2015). Lahmer reported BDG levels were significantly lower in patients with no PCP (86; 30–315 pg/ml) than in patients with a high positive PCP PCR (589; 356–1000 pg/mL; p =0.001) and low positive PCP (398; 297–516 pg/mL; p=0.004) (Lahmer *et al.*, 2017). This approach enhances the diagnosis of PCP in non-HIV patients and may offer clinicians the confidence to treat or withhold treatment in patients where the clinical diagnosis is unclear.

### 10.5 Combined biomarkers for diagnosis of invasive aspergillosis

In our research we demonstrated 100% performance in the use of combined Galactomannan and PCR for the diagnosis of invasive aspergillosis in haemato-oncology patients. The strategy of combined GM-EIA has also been demonstrated to have high performance in other studies on blood and BAL specimens (Bellanger *et al.*, 2011; Torelli *et al.*, 2011; Eigl *et al.*, 2015; Boch *et al.*, 2016). *Aspergillus* PCR and GM-EIA from serum have equivalent performance (Pini *et al.*, 2014). Therefore combining the two tests and applying criteria of one of the two tests being positive when screening patients for IA is effective at detecting biomarkers of disease. Bellanger and colleagues reported the use of PCR and GM-EIA from serum in 16 cases of proven and probable IA. For 56.3% (9/16) of the samples both tests were simultaneously positive, all in

probable patients (Bellanger *et al,* 2011). Therefore this could have supported the diagnosis by providing increased confidence in the positivity of each test. For 18.8% of the samples PCR was positive without GM-EIA positivity and 25% were GM-EIA positive without PCR positivity at the same time (Bellanger *et al,* 2011).

The complicating issue when utilising dual biomarkers for the diagnosis of IA, is that only one positive test still leaves a degree of uncertainty in the significance of the result. In a multicentre study by Boch and colleagues it was demonstrated that a single positive test when using GM and PCR on blood had a sensitivity of 22%, specificity of 97% a PPV of 89% but an NPV of 49% (Boch *et al.*, 2016). In an animal model study utilising PCR and GM-EIA for the detection of *Aspergillus* from blood the highest performance was obtained when using the two tests in combination with an AUC of 0.95, however authors reported that no dual combination could entirely exclude disease but accepting one of two tests as positive had a sensitivity of 100% (White *et al.*, 2016). The cost effectiveness of utilising two biomarkers for screening patients at risk of IA needs to be considered, as twice-weekly screening with the two assays will contribute to an increase in the cost per test. In a randomised control trial Macesic demonstrated no significant difference between the control group and the intervention group receiving GM-PCR screening (Macesic *et al.*, 2017). There was a reduction in mortality from 14.7% to 10.1% (p=0.57) that was close to, but showed no statistical significance. In the cost analysis the authors estimated a cost per lifesaving of £242,661 at 180 days (Macesic *et al.*, 2017). The utilisation of biomarkers for screening does not reduce the overall cost burden but does have an impact on survival and attributed cost per life year. The authors concluded the GM-PCR testing is not cost sparing but cost effective (Macesic *et al.*, 2017). In an open label controlled trial across 13 centers using GM-PCR based strategy the time to diagnosis was significantly reduced (p=0.027) from 20 to 13 days using biomarkers compared within the control arm (Aguado *et al.*, 2015).

There is clear evidence that a dual biomarker diagnostic strategy would be beneficial for the screening and diagnosis of patients at high risk of IA, however, implementation of this process in the current clinical environment is challenging from budgetary and logistical standpoints. In our centre we chose to utilise an alternate strategy, using PCR on BAL alongside GM-EIA testing from BAL and serum. This is a similar approach to one investigated by Boch and colleagues who demonstrated the optimal combination of biomarker performance being PCR and GM-EIA from BAL and BDG from blood (Boch *et al.*, 2016). Boch demonstrated sensitivity, specificity, PPV and NPV of 92, 93, 94 and 90% respectively with this triple testing strategy (Boch *et al.*, 2016). This

approach is limited by the fact that a BAL sample is required as part of the testing algorithm but does target the use of biomarkers to specific specimens based on test performance and in combination offers a profile for the diagnosis of IA. Torelli and colleagues reported 94.1% concordance for positive results from BAL for PCR and GM-EIA and 98.6% concordance for negative results (Torelli *et al.*, 2011). Therefore employing a testing strategy for BAL with two biomarkers and maintaining the use of BDG or GM-EIA from serum appears to be a reserved but suitable alternative approach until a time at which the use of PCR can be introduced. Our future aims are to audit our local service and summarise performance of this testing strategy following one year of established clinical testing.

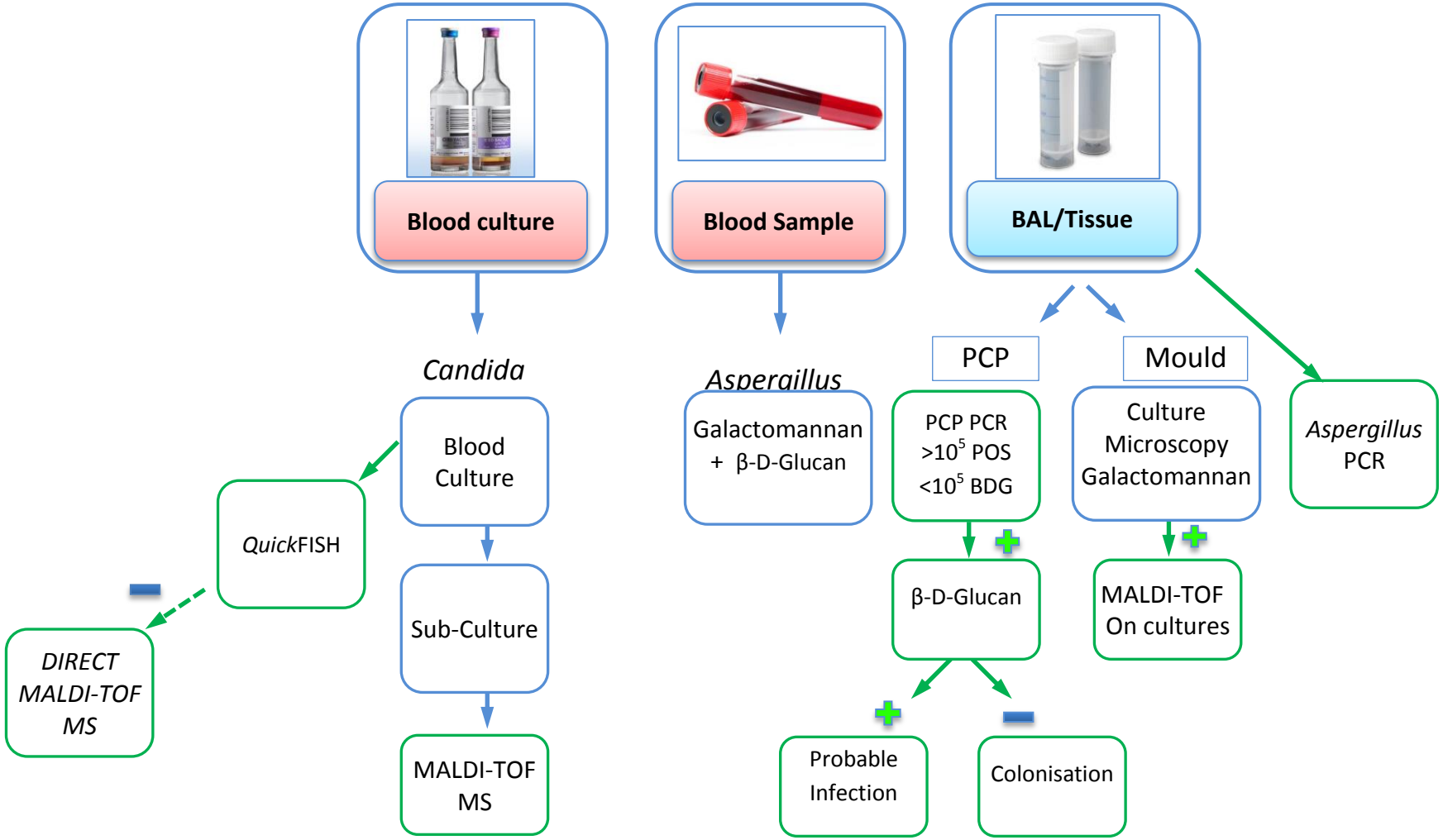



Figure 10.2 Routine Diagnostic Service in the new HSL microbiology laboratory – 2017.

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# Evaluation of culture processing methods and the Bruker MALDI BioTyper™ 3 database for the identification of yeasts

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## INTRODUCTION

Conventional methods for yeast identification, including culture and biochemical profiling, have a turn around time of 24-72 hours. Commercially available biochemical assays have restricted databases leading to misidentification of rarer cryptic species [1,2].

The recent introduction of MALDI-TOF mass spectrometry (MS) into the routine diagnostic setting has enabled yeast identification in under 10 minutes from culture. This study aimed to evaluate three culture processing methods and the Bruker BioTyper™ 3 database, compared with conventional phenotypic and ITS1&2 rRNA sequencing methods for yeast identification. Once validated prospective MALDI-TOF yeast identifications were collected over a one year period.

## MATERIALS & METHODS

**Yeast isolates**; *n* = 488 including; 191 retrospective isolates (validation) and 297 prospective isolates.

**Conventional identification**

191 validation isolates; Sub-culture on CHROMagar™ Candida followed by Auxacolor™ 2 (BIORAD) and cornmeal agar phenotyping for non-albicans species.

**ITS rRNA genotypic identification (Gold standard)**;

DNA extraction – mechanical lysis and Promega Wizard kit. ITS amplification performed using primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'). Sequencing; BigDye® Terminator v3.1 Cycle Sequencing method on an Applied Biosystems 3130 platform.

**MALDI-TOF pre-processing**;

- **Direct smear analysis** - small amount of biomass smeared onto the target.
- **Direct on plate FA extraction** -1µl 100% formic acid (FA) added to direct smear and allowed to dry prior to matrix.
- **Conventional FA extraction** – as per standard protocol.

**MALDI-TOF analysis.**

1µl α-Cyano-4-hydroxy-cinnamic-acid matrix, Microflex™ LT platform (Bruker Daltonics) analysing spectra in the 2000-20,000 m/z range standard settings. Bruker Biotyper™ log score thresholds for secure species >2.0. Re-analysis of spectra was also performed lowering the species acceptable threshold to >1.9.

## RESULTS

**MALDI-TOF validation.** The Bruker Biotyper™ identified 96.5%(184/191) of isolates, demonstrating 100% agreement (184/184) with ITS rRNA sequencing. 3.5% (7/191) of isolates were not identified despite acquiring good MS spectra (Table 1).

**Table 1. Conventional misidentifications and MALDI-TOF 'No identification' compared to ITS rRNA results**

| Conventional | n | MALDI-TOF | ITS rRNA |
|---|---|---|---|
| *C. parapsilosis* | 3 | *C. orthopsilosis* | *C. orthopsilosis* |
| *C. krusei* | 1 | *C. lambica* | *C. lambica* |
| *C. albicans* | 1 | *C. dubliniensis* | *C. dubliniensis* |
| *C. tropicalis* | 1 | *Pichia cactophila* | *Pichia cactophila* |
| *C. glabrata* | 1 | *C. nivariensis* | *C. nivariensis* |
| *C. guilliermondii* | 4 | NO ID | *Candida fermentati* |
| *C. pelliculosa* | 1 | NO ID | *Candida fabianii* |
| *G. candidum* | 1 | NO ID | *Unresolved* |
| *C. famata* | 1 | NO ID | *D. nepalensis* |

**Conventional method evaluation.** Conventional methods identified 93% (177/191) of isolates correctly compared to ITS rRNA sequencing. 7% (14/191) were misidentified by conventional methods (Table 1).

**Pre-processing methods;** On the first attempt at identification the following MALDI-TOF success rates were observed ;
(**>2.0 />1.9 log score)**

- **Dir**ect smear analysis - 0% (0/184) /both thresholds
- Direct on plate FA - 42% (78/184)/66% (121/184)
- Conventional FA extraction - 86% (158/184)/92% (170/184)

**Prospective evaluation of MALDI-TOF** Between January 2012-January 2013 297 yeast isolates were identified by the diagnostic service using MALDI-TOF. 99.7% (296/297) isolates identified successfully using a log score >1.9. One isolate, *Pichia fabianii,* did not identify using MALDI-TOF MS requiring reference laboratory testing.

Table 2. details the yeast species identified by the Bruker Biotyper™ in this study including retrospective and prospective isolates.

**Table 2. Yeast species identified by the Bruker Biotyper ™**

| Species | n | Species | n |
|---|---|---|---|
| *Candida albicans* | 192 | *Cryptococcus neoformans* | 5 |
| *Candida glabrata* | 91 | *Candida dubliniensis* | 5 |
| *Candida parapsilosis* | 84 | *Candida lusitaniae* | 4 |
| *Candida tropicalis* | 36 | *Candida lipolytica* | 2 |
| *Candida guilliermondii* | 17 | *Candida nivariensis* | 2 |
| *Candida krusei* | 11 | *Magnusiomyces capitatus* | 1 |
| *Saccharomyces cerevisiae* | 11 | *Pichia cactophila* | 1 |
| *Candida kefyr* | 8 | *Candida pelliculosa* | 1 |
| *Candida orthopsilosis* | 5 | *Trichosporon beigelii* | 1 |
| *Candida famata* | 1 | *Candida lambica* | 1 |

## CONCLUSIONS

MALDI-TOF MS and the Bruker BioTyper™ 3 software is an accurate method for the identification of clinically relevant yeasts. The Bruker BioTyper™ 3 database contains >100 yeast species and is capable of identifying cryptic species such as *C. orthopsilosis* where current conventional phenotypic methods cannot. Misidentification did not occur. If the Bruker Biotyper™ cannot identify a yeast genotypic methods should be used, as conventional phenotypic methods will give an incorrect result. In routine practice this is a rare occurrence.

FA extraction of proteins is crucial and conventional FA extraction is recommended for yeasts, although some success is possible with direct on plate FA extraction. With an estimated reagent cost of £0.30 per sample MALDI-TOF contributes to considerable cost savings and reduces the turn-around time for yeast identification by up to 72 hours.

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# Evaluation of sensitivity, specificity and potential clinical impact of Yeast Traffic Light™ PNA FISH for the rapid identification of yeasts directly from blood cultures

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## INTRODUCTION

Candidaemia carries a high mortality[1], with significantly worse outcomes resulting from delay in diagnosis and appropriate antifungal therapy[2]. Current laboratory techniques result in identification of candida species from blood cultures in 24-72 hours.

Peptide Nucleic Acid Fluorescent In – Situ Hybridisation (PNA FISH) employs PNA probes designed to complement species specific rRNA sequences. The Yeast Traffic Light PNA FISH assay ™ (AdvanDx, Copenhagen) uses species specific probes labelled with different fluorescent dyes to allow visualisation of yeasts within a blood film using fluorescent microscopy. This allows for speciation directly from a positive blood culture smear. The assay is capable of distinguishing between *Candida albicans, C.parapsilosis, C. tropicalis, C. glabrata and C. krusei*, the species which account for 90% of candidaemias [3]. Turnaround time for a result is approximately 90 minutes.

Our objective was to evaluate the PNA FISH assay's sensitivity and specificity, and to estimate the potential clinical impact and cost saving offered by routine use in the clinical setting.

## MATERIALS & METHODS

**Yeast isolates:** 52 isolates of candida species from blood cultures, previously identified by Auxacolor were selected for inclusion in the study.

**Spiking blood cultures:** Approximately 200 cfu were spiked into 5 day negative blood culture bottles and incubated on a BACTEC® (BD,Oxford) platform until positivity was detected.

**PNA FISH method**: One drop of blood was added to each PNA FISH slide and heat fixed with fixation solution for 20 minutes. A drop of PNA Traffic Light™ probe was added to each slide and hybridised by heating at 55°C for 30 minutes. The slides were then washed for 30 minutes in a wash solution to remove excess unbound probe. A mounting solution was added and a coverslip applied. A control slide was prepared each time the method was performed. The slides were then viewed using fluorescent microscopy. Visualisation of green fluorescence indicated the presence of *C. albicans* or *C. parapsilosis*, red fluorescence indicated *C. glabrata* or *C .krusei* and yellow fluorescence indicated *C. tropicalis*. The researcher performing PNA FISH was blinded to the identity of the yeast.

**Clinical impact assessment** : A retrospective review of clinical notes of patients from whom the yeasts had been isolated was carried out.. Predicted choice of antifungal therapy was estimated on the basis that identifying albicans, parapsilosis, or tropicalis would have resulted in commencing fluconazole therapy, and identifying glabrata or krusei would have prompted the use of caspofungin as initial therapy.

## RESULTS

**Results by species - colour visualised on fluorescent microscopy**

| Routine ID | Number of isolates | C. albicans/C. parapsilosis (green) | C. .tropicalis (yellow) | C. glabrata/C. krusei (red) | Negative |
|---|---|---|---|---|---|
| *C albicans* | 17 | 17 | – | – | – |
| *C parapsilosis* | 10 | 10 | – | – | – |
| *C tropicalis* | 4 | – | 4 | – | – |
| *C krusei* | 4 | – | – | 4 | – |
| *C glabrata* | 8 | – | – | 8 | – |
| *C glabrata + tropicalis* | 1 | – | 1 | – | – |
| *C albicans + tropicalis* | 1 | 1 | 1 | – | – |
| *C guillermondii* | 2 | – | – | – | 2 |
| *C lusitaniae* | 1 | – | – | – | 1 |
| *C pelliculosa* | 1 | – | – | – | 1 |
| *B capitatus* | 1 | – | – | 1 | – |
| *C neoformans* | 2 | – | – | 1 | 1 |

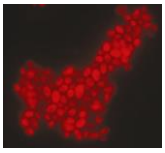
**Sensitivity by probe**

| *C. albicans/C. parapsilosis* (green) | *C. .tropicalis* (yellow) | *C. glabrata/C. krusei* (red) |
|---|---|---|
| 100% (28/28) | 100% (6/6) | 92.3% (12/13) |

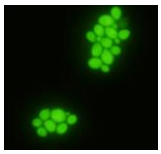
**Specificity by probe**

| Non *C.albicans/C. parapsilosis* (green fluorescence not produced) | Non *C.tropicalis* (yellow fluorescence not produced) | Non *C.glabrata/C.krusei* (red fluorescence not produced) |
|---|---|---|
| 100% (24/24) | 100% (46/46) | 94.8 % (37/39) |

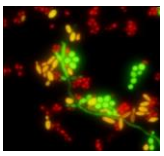
Results demonstrated high sensitivity and specificity rates for the species the assay detects. Two false positives for the glabrata/krusei probe were observed (red fluorescence) with one *Cryptococcus neoformans* culture and *Blastoschizomyces capitatus*. On repeat testing no red fluorescence was observed with the *C. neoformans,* and very faint red fluorescence was observed for the *B. capitatus*. One C. glabrata was missed in a mixed culture



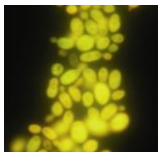
Candida glabrata/krusei



Candida albicans/parapsilosis



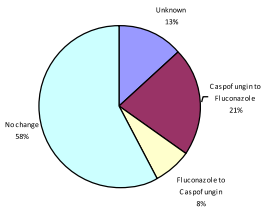
Mixed smear



Candida tropicalis

**Clinical impact**: Predicted change in choice of initial antifungal therapy if speciation had been available within 4 hours of positive blood culture flagging

| | |
|---|---|
| NO CHANGE | 32 |
| UNKNOWN | 5 |
| FLUCONAZOLE TO CASPOFUNGIN | 4 |
| CASPOFUNGIN TO FLUCONAZOLE | 11 |




## CONCLUSIONS

PNA – FISH is a rapid and highly sensitive and specific technique for identification of candida species directly from blood cultures. This has the potential to alter initial antifungal therapy , which could significantly reduce the use of echinocandins , with a corresponding cost saving. Prospective trials are required to evaluate clinical outcomes and cost benefit.

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2. Kibbler CC et al. Management and outcome of bloodstream infections due to Candida species in England and Wales. J Hosp Infec t 2003 May;54(1):18-24.

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# A comparison of three rapid identification techniques for the identification of yeasts from positive blood cultures: Gram's stain, PNA-FISH and MALDI-TOF

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## INTRODUCTION

Early onset of appropriate antifungal treatment is associated with a reduction in morbidity and mortality in patients with Candidemia[1]. Reducing the time to identification of yeasts from blood cultures, currently 24-72 hours, could improve clinical outcome by influencing accurate choice of antifungal therapy earlier.

The aim of this comparative study was to investigate three rapid laboratory techniques for the identification of yeasts directly from blood cultures in less than 4 hours.

**Gram's stain** - Unique morphological characteristics of yeasts, e.g. true hyphae with *C. albicans* (Figure A), seen within gram's stained blood films may be used to identify species.

**Peptide Nucleic Acid Fluorescent In – Situ Hybridisation (PNA-FISH)** - PNA probes labelled with different fluorescent dyes complement species specific rRNA sequences for *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei* (Figure B).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF)** is a technique measuring biomolecules, including proteins, from samples (Figure C). Spectra are matched against a library of reference spectra to identify unknown organisms.

## METHODS

**Yeast Isolates;** 50 yeast isolates previously implicated in Candidemia were selected and sub-cultured onto CHROMagar™ Candida (BD) Identified was achieved by biochemical profiling using the Auxacolor2 system (BioRad).

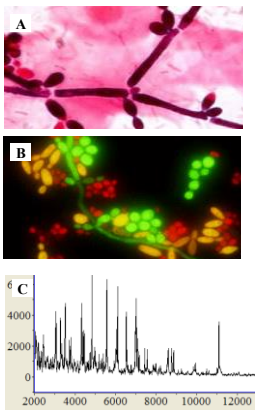
**Simulated Blood Cultures;** ~200 cfu inoculated into five-day negative blood culture bottles, were incubated until flagged positive (BD BacTec™). Sub-culture and Gram's stain confirmed growth and purity.

**Gram's stain;** Blood smears were fixed and standard gram's stain method was applied. Slides were analysed by a health care scientist experienced in examining yeast blood culture slides (4 months). An image gallery of 7 yeasts species was used as an identification aid.

**MALDI-TOF**; Blood culture bottles were vortexed, 1ml aspirated and vortexed for 5 minutes. Bruker Sepsityper™ kit was used for blood culture processing as per manufacturer's instructions. The Bruker formic acid (FA) extraction protocol was used for protein extraction with a 15 minute FA incubation step. 1µl of analyte, inoculated onto the target in triplicates was overlaid with 1µl α-Cyano-4-hydroxy-cinnamic-acid matrix. Spectra acquisition was performed on a Microflex™ LT platform (Bruker Daltonics) with FlexControl software (version 3.0) using default settings. Spectra analysis was performed using MALDI Flex analysis™ software analysing spectra in the 5000-15000 m/z range. Score thresholds; no reliable ID 0.0-1.599, probable genus 1.6-1.8, secure genus, probable species 1.8 - 2.0, highly probable species >2.0.

**PNA FISH**: The AdvanDX PNA Traffic Light™ assay was used as per manufacturers instructions. Green fluorescence - *C. albicans*/*C. parapsilosis*, red fluorescence - *C. glabrata* /*C .krusei* and yellow fluorescence - *C. tropicalis*. (Figure B)

Figure A) *C. albicans* gram stain, Figure B) PNA-FISH with *C. albicans*, *C. tropicalis* and *C. glabrata*, Figure C) MALDI-TOF spectra for *C. glabrata*.




## RESULTS

Table 1 details the frequencies of classification, misclassification and non-recognition for Gram's stain, MALDI-TOF and PNA-FISH. Table 2 presents isolates missed or misidentified by Gram's stain, PNA-FISH and MALDI-TOF

**Table 2. Isolates missed or misidentified by Gram's stain, PNA-FISH and MALDI-TOF**

| Yeast Species | *n* | Gram's stain | | PNA-FISH | *n* | MALDI-TOF | |
|---|---|---|---|---|---|---|---|
| ***C. albicans*** | 17 | *C. tropicalis* | 2 | - | | Missed | 4 |
| | | *C. parapsilosis* | 1 | - | | Genus | 5 |
| | | Missed | 1 | - | | | |
| ***C. tropicalis*** | 4 | *C. albicans* | 1 | - | | Genus | 2 |
| ***C. glabrata*** | 9 | *C. parapsilosis* | 1 | - | | Missed | 3 |
| ***C. krusei*** | 4 | *C. parapsilosis* | 3 | - | | | |
| ***C. parapsilosis*** | 9 | *C. albicans* | 1 | - | | Missed | 2 |
| ***C. guillermondii*** | 2 | *C. glabrata* | 1 | - | | Genus | 2 |
| ***C. lusitaniae*** | 1 | *C. glabrata* | 1 | - | | Missed | 1 |
| ***C. pelliculosa*** | 1 | *C. glabrata* | 1 | - | | Missed | 1 |
| ***C. neoformans*** | 2 | - | | *C. glabrata* | 1 | Missed | 2 |
| ***B. capitatus*** | 1 | - | | *C. glabrata* | 1 | Genus | 1 |

Species names indicate misidentifications, 'Missed' isolates contained within the database but not recognised, 'Genus' a correct confirmed genus identification only but correct species were indicated for all.

Table 3 details cost, turn around time and technical requirement for Gram's stain, PNA-FISH and MALDI-TOF

**Table 3. Cost, turn around time and technical requirement for Gram's stain, PNA-FISH and MALDI-TOF**

| | Gram's stain | PNA-FISH | MALDI-TOF |
|---|---|---|---|
| **Cost per test** | £0.10* | £70.00* | £1.30* |
| **Turnaround time** | 15 minutes | 90 minutes | 90 minutes |
| **Technical requirement** | Experienced[5][7] | Inexperienced[8,11] | Inexperienced[11] |

*Not including NHCS salary costs, [5]Required 3-12 weeks training, [8]Requires 3-12 week training.[8]

## CONCLUSIONS

Gram's stain is inexpensive, rapid and relatively successful. However, almost 25% of isolates were misidentified, including three *C. krusei* identified as *C. parapsilosis,* which would have had a significant impact on treatment choice.

MALDI-TOF is cost effective, rapid and requires minimal technical ability. The assay also has the most extensive database including >100 species. Yet this approach lacks the sensitivity required from a diagnostic assay, most likely associated with inadequate protein extraction from yeasts.

PNA-FISH was very successful at detecting yeast species targeted by the assay and requires little technical expertise. The assay is limited by the range of species it detects and at £70.00 per test is the most expensive.

Until protein extraction methods are improved for MALDI-TOF, PNA-FISH is the most applicable method for rapid identification of yeasts from blood cultures.

1) Tortorano AM *et al* Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur J Clin Microbiol Infect Dis*. 23: 317-322. 2004

**Table 2. Frequencies of classification, misclassification and non-recognition for Gram's stain, MALDI-TOF and PNA-FISH**

| | Subset description | | | Classification results | | | |
|---|---|---|---|---|---|---|---|
| | Species (n) | Isolates (n) | % of total | Correct | Misclassified | Unknown | Success rate (%) |
| (a) Performance total | | | | | | | |
| Gram's stain | 10 | 50 | 100.0 | 36 | 13 | 1 | 72 |
| PNA-FISH | 10 | 50 | 100.0 | 43 | 2 | 5 | 86 |
| MALDI-TOF | 10 | 50 | 100.0 | 28 (38)* | 0 | 12 | 56 (76)* |
| (b) Performance only on species in respective method database | | | | | | | |
| Gram's stain | 7 | 47 | 94 | 36 | 10 | 1 | 76.6 |
| PNA-FISH | 5 | 43 | 86 | 43 | 0 | 0 | 100 |
| MALDI-TOF | 10 | 50 | 100 | 28 (38)* | 0 | 9 | 56 (76)* |
| (c) Performance only on species contained in all three databases | | | | | | | |
| Gram's stain | 5 | 43 | 86 | 34 | 8 | 1 | 79 |
| PNA-FISH | 5 | 43 | 86 | 43 | 0 | 0 | 100 |
| MALDI-TOF | 5 | 43 | 86 | 26 (35)* | 0 | 8 | 60.4 (81.4)* |
| (d) Performance total (including 'unknown' as the correct identification for isolates absent from the database) | | | | | | | |
| Gram's stain | 10 | 50 | 100.0 | 36 | 13 | 1 | 72 |
| PNA-FISH | 10 | 50 | 100.0 | 48 | 2 | 0 | 96 |
| MALDI-TOF | 10 | 50 | 100.0 | 28 (39)* | 0 | 11 | 56 (78)* |

*where confidence scores secured genus identification, 1.600-1.799, the correct species was indicated for all isolates.