New approaches to the detection of echinocandin resistance in *Candida glabrata* in clinical diagnostic laboratories

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy

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September 2019

ACKNOWLEDGEMENTS

Thank you to Dr Lynne Lawrance for an uncompromising belief that I had the ability to produce this work, and for being a constant supporter of everything I've attempted. Thanks also to Dr Robin Thorn for his additional support, suggestions and kind words when things were tough.

Thank you to everyone at the Mycology Reference Laboratory for the collective patience, advice and support throughout the years that have gone into producing this thesis. In particular, Dr Andrew Borman who, despite the daily carnage, enabled me to produce something all my own and provided solidarity in times of need, Dr Elizabeth Johnson for providing me with the platform and support needed to undertake research in a clinical laboratory, PHE for financial support towards tuition fees and Zoë Patterson and Philippa Brown, whose unwavering confidence in my abilities and continual supply of affection and encouragement helped me get through the hardest times.

Thank you to Dr Kate Heesom at the Proteomics facility, University of Bristol for undertaking the LC-MS/MS work.

I dedicate this to my family. Mum, Dad, Nan, Granny, Kylie and Poppy; I have only achieved this through my wish to make you proud. Your unquestioning faith in what I could be and what I could achieve made it all possible. Finally, Cian it's me and you, stuck like glue, soul to soul, home from home, perfect two, that's me and you. Without your love and support I would never have started or indeed finished this work.

ACRONYMS and ABBREVIATIONS

2D PAGE – 2-dimensional polyacrylamide gel electrophoresis

AIDS – acquired immunodeficiency syndrome

ANF – anidulafungin

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

BLASTn – basic local alignment search tool for nucleotides

BSI – blood stream infection

bp – base pair

CBS – Centraalbureau voor Schimmelcultures, Westerdijk Institute culture collection, The Netherlands

CCI – composite correlation index

CDCP - Centers for Disease Control and Prevention

CFU – colony forming unit

CLSI – Clinical Laboratory Standards Institute

CSP – caspofungin

DMSO – dimethyl sulphoxide

DNA – deoxyribonucleic acid

dNTP – dinucleotide triphosphate

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FASTA – fast all consensus format for DNA/RNA sequence analysis

FDR – false discovery rate

HAI – hospital-acquired infection

HCCA – α-cyano-4-hydroxycinnamic acid

HIV – human immunodeficiency virus

IVDU – intravenous drug use/user

IQC – Internal quality control

LC-MS/MS – liquid chromatography mass spectroscopy /mass spectroscopy

LN2 – liquid nitrogen

MALDI-TOF MS – matrix assisted laser desorption ionisation time of flight mass spectroscopy

MCF - micafungin

MFC – minimum fungicidal concentration

MIC – minimum inhibitory concentration

MRL – Mycology Reference Laboratory

MRLDB – Mycology Reference Laboratory internally curated MALDI-TOF MS database

MSP – main spectrum profile

MW – molecular weight, usually in Daltons (Da)

NCCLS – National Committee for Clinical Laboratory Standards

NCPF – National Collection of Pathogenic Fungi

NGS – next generation sequencing

NHS – National Health Service

OS – organic solvent

PCA – principal component analysis

PCR – polymerase chain reaction

PHE – Public Health England

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

rpm – revolutions per minute

RPMI – RPMI tissue culture medium supplemented with 10% glucose

SA – sinapinic acid

SABC – Sabouraud's agar with chloramphenicol

SNP – single nucleotide polymorphism

TFA – trifluoroacetic acid

WGS – whole genome sequencing

Units of Measurement

Da – Dalton, unified atomic mass unit

mg – milligram

µg – microgram

mL – microlitre

µM – micro Molar

m/z – mass/ charge ratio

Relative Centrifugal Force (g) = 1.12 x Radius x $(rpm/1000)^2$

ABSTRACT

Candidaemia is widely reported as the fourth most common form of bloodstream infection worldwide. Reports of cases of candidaemia whilst patients are in receipt of antifungal therapy are increasing, and this is especially relevant as prescribing practices change and develop. Given the elevation of echinocandin antifungal agents as first line treatment options over the triazole antifungal agents, and the increased use of echinocandin antifungal agents as a prophylactic choice, it is important to apply suitable surveillance in order to counteract potential difficulties which may arise from the emergence of resistance to the echinocandin class of antifungal agents.

This study has designed and created a suitable assay for the specific detection of *FKS* gene mutations in *Candida glabrata* to indicate resistance to echinocandin antifungal agents using a pyrosequencing-based platform in the clinical diagnostic laboratory. There exists the potential for this rapid molecular detection system to be used as a screening tool which would help provide clinicians with essential information required to make appropriate and accurate therapeutic decisions for the management of bloodstream infections. This assay allows the reporting of these results within 4 hours of isolation, greatly improving reporting times in the clinical laboratory. This study also provided data to support evidence of a continued low level of echinocandin resistance prevalent in *C. glabrata* in the United Kingdom.

This study has assessed the potential of proteomic approaches, using LC- MS/MS and MALDI-TOF MS to indicate antifungal resistance, as demonstrated by *C. glabrata* with the echinocandin antifungal agents, by the identification of and the changing patterns in protein presence, absence or relative abundance. This study has solely focused on using techniques that are realistically accessible to a diagnostic microbiology laboratory to maintain an indication of true clinical impact.

No readily identifiable or reproducible patterns in proteomic variation between echinocandin resistant and echinocandin susceptible isolates were found. However, the importance of continuing to adapt and modify the capabilities of the modern clinical diagnostic laboratory in an era of increasing antimicrobial resistance is highlighted.

Word Count: 330

FIGURES AND TABLES

Figures

		Page Number
1.	A prepared MALDI-TOF MS target plate	37
2.	Sanger sequencing trace for *C. glabrata* NCPF 8714 *FKS1*	46
3.	BLASTn matches for *FKS1* primer	47
4.	Sanger sequencing trace for *C. glabrata* NCPF 8714 *FKS2*	48
5.	BLASTn matches for *FKS2* primer	49
6.	Pyrosequencing analysis report for *FKS* mutant	51
7.	MALDI-TOF MS protein spectra for *C. glabrata* isolates NCPF 8745 and NCPF 8814	66
8.	Example of spectra generated by an increased mass detection window in the FlexAnalysis software	68
9.	Example spectra generated when using sinapinic acid as the matrix	70
10.	An example MALDI-TOF MS spectrum for *C. glabrata* showing detection parameters of spectra acquisition	74
11.	Comparative spectra generated for two isolates of *C. glabrata* by MALDI-TOF MS	75
12.	Example database match for an uncharacterised clinical isolate of *C. glabrata*	76
13.	A principal component analysis (PCA) of spectral data for NCPF 8745 and NCPF 8814	78
14.	A composite correlation index (CCI) to compare spectral data for NCPF 8745 and NCPF 8814	79
15.	A principal component analysis (PCA) for four unrelated isolates of *C. glabrata* with *FKS1* and *FKS2* mutations	80
16.	A composite correlation index (CCI) to show the spectral relationship between four unrelated isolates of echinocandin resistant *C. glabrata*	81
17.	MALDI-TOF MS spectra generated for NCPF 8814 over a concentration gradient of caspofungin	83

18. MALDI-TOF MS spectra generated for NCPF 8814 at different time points during incubation in 1 μg/mL of caspofungin 84

19. MALDI-TOF MS spectra for NCPF 8814 over a concentration gradient of caspofungin identifying potential marker of resistance 88

Tables

Page Number

1. Details for all isolates demonstrating echinocandin resistance included in this study 24

2. Echinocandin breakpoint values for the interpretation of susceptibility in *C. glabrata* by microbroth dilution as recommended by CLSI. 28

3. Pyrosequencing primers used for detection of *FKS1* and *FKS2* mutations 30

4. Sequence and codon positions of *C. glabrata FKS1* region 32

5. Sequence and codon positions of *C. glabrata FKS2* region 32

6. Minimum inhibitory concentration (MIC, μg/mL) of caspofungin as demonstrated by E-test for revived NCPF isolates 42

7. Minimum inhibitory concentration (MIC, μg/mL) of echinocandin for all isolates used during this study between July 2015 - July 2018 44

8. Pyrosequencing results for NCPF isolates of *C. glabrata* 52

9. Sub-culture and resulting macro-colonies of NCPF isolates exhibiting decreased susceptibility to anidulafungin 54

10. *FKS* results for clinical isolates showing *in vitro* resistance 56

11. The number of whole proteins predicted from peptide sequence spectra generated by LC-MS/MS 60

12. Predicted unique proteins in echinocandin resistant isolate, NCPF 8814 61

13. Proteins predicted by LC-MS/MS from NCPF 8814 which are only present in MALDI-TOF MS extraction 62

14. MALDI-TOF MS database matches for an uncharacterised clinical isolate of *C. glabrata* 77

15. Results of growth experiments for macro-broth cultures 86

16. The presence or absence of a potential marker of resistance as detected by MALDI-TOF MS in a selection of isolates 89

CONTENTS

i. Acknowledgments

ii. Acronyms, Abbreviations and Units of Measurement

v. Abstract

vi. List of Figures and Tables

Page Number

1. INTRODUCTION **1**

1.1 *Candida glabrata* 2

1.2 Bloodstream infections (BSI) 4

1.3 The echinocandin class of antifungal agents and their use in the treatment of *C. glabrata* 6

1.4 Diagnostic testing in the clinical mycology laboratory 9

1.5 Genomic detection of resistance markers in *C. glabrata* 12

1.6 Detection of proteins for the identification and classification of microorganisms 14

1.7 MALDI-TOF MS and the identification of fungi 17

1.8 Study aim 20

1.8.1 Study objectives 21

Page Number

2. MATERIALS AND METHODS 23

2.1 Collection/selection of *C. glabrata* isolates 23

2.2 Susceptibility testing of *C. glabrata* isolates 25

2.2.1 Epsilometer (E-test) susceptibility testing 25

2.2.2 Microbroth dilution susceptibility testing 25

2.2.3 Macro-broth dilution susceptibility testing 27

2.2.4 Interpretation of susceptibility testing results 27

2.2.5 *In vitro* induction of resistance 29

2.3 Genomic detection of echinocandin resistance 30

2.3.1 Primer design for amplification of *FKS* mutations 30

2.3.2 Pyrosequencing and rapid detection of *FKS* mutations 31

2.4 Detection of proteins to indicate resistance by LC-MS/MS 33

2.4.1 Preparation of samples for LC-MS/MS 33

2.4.2 Extraction protocol for LC-MS/MS MALDI-TOF MS analysis 34

2.4.3 LC-MS/MS data analysis 34

2.5 Preparation and analysis of samples by MALDI-TOF MS 36

2.5.1 Basic protein extraction 36

2.5.2 Extraction of proteins after antifungal exposure 37

2.5.3 MALDI-TOF MS analysis 38

2.5.4 Creation of main spectrum profiles (MSP) for inclusion in the MALDI-TOF MS database 38

2.6 Data analysis 39

2.6.1 Genomic analysis 39

2.6.2 Proteomic analysis 40

2.7 Ethical approval and risk assessment 41

Page Number

3. RESULTS 42

3.1 Detection of genetic markers of resistance 42

3.1.1 Collection/Selection of isolates and susceptibility testing 42

3.1.2 Primer design and suitability for analysis of *FKS* regions 45

3.1.3 Pyrosequencing of *FKS* regions 50

3.1.4 *In situ* induction of resistance 52

3.1.5 Application to clinical isolates demonstrating phenotypic resistance 55

3.2 Detection of proteins by LC-MS/MS that may indicate resistance 57

3.2.1 LC-MS/MS analysis of protein extracts 57

3.2.2 Detection of predicted proteins by LC-MS/MS using MALDI-TOF MS 65

3.2.3 The effect of widening mass detection window in MALDI-TOF MS 67

3.2.4 The use of a different matrix for the ionisation of high molecular weight proteins 69

3.3 Protein analysis by MALDI-TOF MS 71

3.3.1 Creating an MSP for resistant isolates 71

3.3.2 The effect of growth in the presence of echinocandin on MALDI-TOF MS spectra 82

3.3.3 The effect of increasing the fungal load and sample volume 85

Page Number

4. DISCUSSION 90

4.1 Analysis of *FKS* mutation detection as a predictor of resistance 92

4.2 Re-evaluation of MIC data in the context of changing laboratory protocols 98

4.3 The use of proteomic approaches for the detection of resistance 100

4.3.1 LC-MS/MS in the clinical diagnostic mycology laboratory 101

4.3.2 MALDI-TOF MS in the clinical diagnostic mycology laboratory 105

4.4 The role of research in the clinical mycology laboratory 110

4.5 Critical evaluation of research undertaken as part of this thesis 111

4.6 Potential future research opportunities arising from the research in this thesis 116

4.7 The future of resistance detection in the clinical mycology laboratory 118
4.8 Conclusions 120

5. References **123**
6. Appendices **149**

1. INTRODUCTION

Fungi are eukaryotic microorganisms that inhabit almost every known ecological niche on earth. Fungi are distinct from both plants and animals sharing characteristics with both yet existing within a taxonomic kingdom of their own. Fungal cells incorporate a rigid cell wall mostly comprised of chitin and glucan, directly contrasting with animal cells that do not have cell walls, and plants that have cellulose as a major cell wall component. A further distinction can be made at the cellular level by the presence of a cell membrane comprised of ergosterol, a variation when compared to animal cell membranes which tend to contain cholesterol (Gow, Latge and Munro, 2017; Lv, Yan and Jiang, 2016). Fungi are heterotrophic and as such, unlike plants, are unable to manufacture their own carbon sources. This means they play a crucial role in the cycling of organic matter and nutrients within the environment (Treseder and Lennon, 2015). Finally, fungi exhibit much simpler structure than both plants and animals, forming either singular, filamentous strands known as hyphae or a single independent cell, or blastospore. Many fungi that exist in single cell form, often termed yeasts, reproduce by budding progenitor cells from an individual parent cell. The bud may detach, or it may remain *in situ* and begin the formation of a chain of cells. Continued elongation of cells in this way results in the production of pseudohyphae, an intermediate form of morphology between yeasts and true fungal hyphae (Noble, Gianetti and Witchley, 2017).

Hawksworth and Lücking (2017) suggest that potential global fungal diversity indicates there are somewhere between 2.2 to 3.8 million species of fungi, with 120,000 currently accepted species. Similarly, recent advances in high-throughput genetic analyses has also helped to demonstrate great diversity within the fungal kingdom (Nilsson *et al*., 2019; Peay, Kennedy and Talbot, 2016), further highlighting the importance of fungi

in many ecologically important roles such as nutritional recycling and decomposition of materials in the environment (Johnston, Park and Smissen, 2017; Peay, Kennedy and Talbot, 2016). A relatively small proportion of the known fungal kingdom has been associated with human disease and the majority of those are only capable of causing infection in immunocompromised individuals (Enoch *et al*., 2017; Diekema *et al*., 2012). However, many fungi are often considered opportunistic pathogens and demonstrate an ability to exploit any available nutrient sources (Borman *et al*., 2018b; Enoch *et al*., 2017). Infections caused by fungal organisms are often classified with consideration for the initial focus of infection as superficial, subcutaneous and systemic (Richardson and Warnock, 2003). It is probable that changes in patient demographics and likely risk factors for infections with fungal organisms has helped to increase the potential agents of fungal infection. Such factors include: increases in lifestyle-related diseases such as diabetes (Smyth *et al*., 2018); ageing populations (Barchiesi *et al*., 2017); underlying conditions such as HIV/AIDS (Limper *et al*., 2017) or haematological malignancies (Miceli *et al*., 2017); and invasive surgical procedures (Brown *et al*., 2018; Horn *et al*., 2017). Thus, the potential for fungi to cause infection in humans remains a critical and increasingly relevant consideration for clinicians and diagnosticians alike (Enoch *et al*., 2017).

1.1 *Candida glabrata*

Nakaseomyces glabrata is a unicellular fungus which until recently was known as *Candida glabrata* (Borman and Johnson, 2018a; Angoulvant, Guitard and Hennequin, 2016; Gabaldón and Carreté, 2016). Whilst recognising the importance of taxonomic revision and development, this thesis retains the name *C. glabrata* throughout given the

familiarity of this nomenclature in clinical diagnostics and for consistency with publications and research produced during the timeline of this study.

As reviewed by Rodrigues, Silva and Henriques (2014), *C. glabrata* is a species of haploid, unicellular fungi, often termed yeast, which is relatively non-pleomorphic and in which no mating activity has been observed. The single cells, or blastospores of *C. glabrata* have the ability to colonise the mouth, oesophagus, intestine and vaginal mucosal surfaces as part of the commensal biota and the species lacks many of the virulence factors which have been identified in other *Candida* species, such as hyphal growth formation (Noble, Gianetti and Witchley, 2017; Cavalcanti *et al*., 2015), or the ability to secrete tissue-degrading enzymes such as proteases (Rapala-Kozik *et al*., 2018). Also, in contrast to other *Candida* species, many of the recognised risk factors for fungal infection such as diabetes, age and malignancies have been found to be unreliable predictors for infection with *C. glabrata* (Smyth *et al*., 2018).

Yet, patterns in hospital acquired infections (HAI) have shown *C. glabrata* to be a highly opportunistic pathogen of the urogenital tract and the bloodstream (Cleveland *et al.*, 2012). There are reports of *C. glabrata* being implicated in up to 15% of cases of bloodstream infections, especially in the elderly (Barchiesi *et al.*, 2017), those infected with HIV (Limper *et al*., 2017), or with a background of intravenous drug use (IVDU) (Barter *et al*., 2019).

Moreover, specifically with respect to HAIs, patients receiving broad-spectrum antimicrobials, those admitted to intensive care units or who experience prolonged hospitalisation, in-dwelling catheters or recent abdominal surgery (CDCP, 2017; Vallabhaneni *et al*., 2015; Sardi *et al*., 2013; Pfaller *et al*., 2007). Clearly, evidence such as this demonstrates that *C. glabrata* is responsible for a considerable level of observable pathogenicity in humans.

Potential virulence factors that may support *C. glabrata* as a human pathogen include its ability to growth at 37°C (Salvadó *et al*., 2011). *C. glabrata* also appears to exhibit a high level of tolerance to biochemical stress, in particular those characteristics which are related to immune system activation within the host (Kasper, Seider and Hube, 2015). Such properties may be further enhanced by the demonstration of a resistance to starvation which allows the survival of *C. glabrata* blastospores within macrophages following engulfment (Seider *et al*., 2011). Furthermore, the identification of a series of adhesins, cell surface molecules that play a role in cell attachment, and in particular those encoded for by epithelial adhesion genes (EPA), provides *C. glabrata* with the ability to form aggregates on surfaces as demonstrated by atomic force microscopy (Valotteau *et al*., 2019). The ability to form clusters of cells in this way offers *C. glabrata* the ability to produce biofilms thereby providing an additional pathogenic characteristic for this organism. This is of particular importance, and often an additional complication in the clinical setting, where medical devices such as in-dwelling urinary or intravenous catheters are used (Rodrigues *et al*., 2017; Gabaldón and Carreté, 2016).

Additionally, *C. glabrata* has demonstrated an ability to re-organise certain cell wall components, in particular chitin, in response to stresses from its environment, and this has been indicated as having a role in promoting the persistence of *C. glabrata* in the intestinal tract despite the application of antifungal treatment (Vallabhaneni *et al*., 2015).

1.2. Bloodstream infections (BSI)

Infections of the bloodstream (BSI) are a frequent cause of hospitalisation and mortality in both healthy and immunocompromised patients. A recent meta-analysis indicated that BSIs were associated with levels of mortality as high as 50% at one-year post

infection (McNamara *et al*., 2018). The antimicrobial management of patients with BSIs is considered time critical, and in an era of increasing antimicrobial resistance, the accurate and rapid identification of the agent of infection is essential to successful clinical resolution (Poole, Kidd and Saeed, 2018).

Candidaemia is the term used to describe a bloodstream infection (BSI) where the causative organism is a single-celled fungus from the taxonomic group of *Candida* spp., and is widely reported as the fourth most common form of BSI worldwide and presents a considerable, continuing challenge to modern medicine (Pappas *et al*., 2018; Vallabhaneni *et al*., 2015; Magill *et al*., 2014; Cleveland *et al*., 2012: Pfaller *et al*., 2011c; Vincent *et al*., 2009; Wisplinghoff *et al*., 2004). An increase in resistance to established antifungal agents and changing patient demographics are helping to widen the spectrum of species able to cause infection (Deorukhkar, Saini and Mathew, 2014; Guinea, 2014; Pfaller *et al*., 2014b; Diekema *et al*., 2012; Lockhart *et al*., 2012). The appropriate use of antifungal agents is essential for successful clinical outcomes, helping to reduce the burden of emergent resistance and financial strain upon healthcare providers (Mencarini *et al*., 2018; Neoh *et al*., 2018; Jensen, 2016; Ashbee *et al*., 2014; Perez *et al*., 2013).

The epidemiology of candidaemia varies geographically, but *C. glabrata* is consistently reported as the second or third most frequently indicated fungal cause (Astvad *et al.*, 2018; Klingspor *et al*., 2018; Mencarini *et al*., 2018; da Matta *et al*., 2017; Hou *et al*., 2017; Klotz *et al*., 2016; Marcos-Zambrano *et al*., 2014). The retrospective analysis undertaken by Klingspor *et al*. (2018) highlighted that *C. glabrata* remained unchanged as the second most common cause of candidaemia in Sweden over a ten-year period, with 19.7% of all *Candida* species isolated from blood cultures being identified as *C.*

glabrata. Likewise, studies from Latin America have also indicated an increase in BSIs where *C. glabrata* is the main aetiologic agent (da Matta *et al*., 2017).

1.3 The echinocandin class of antifungal agents and their use in the treatment of *C. glabrata*

The use of triazole antifungal agents, in particular fluconazole, as first line treatment options has been considered acceptable for all forms of candidiasis, including BSIs. (Bassetti *et al*., 2018; Ostrosky-Zeichner *et al*., 2003). However, as reviewed by Berkow and Lockhart (2017), clinical resistance to fluconazole has been reported in many settings and with many *Candida* spp. Consequently, *C. glabrata* has also been shown to demonstrate well-established mechanisms of triazole resistance, and many of these mechanisms confer resistance to other triazole agents such as itraconazole, posaconazole and voriconazole (Pfaller et al., 2005; Pfaller *et al*., 2004). In response, investigations into the properties of novel antifungal compounds and targets led to the introduction of the echinocandin class of agents (Walsh *et al*., 2000).

The echinocandin class of antifungal agents currently consists of caspofungin (CSP), anidulafungin (ANF) and micafungin (MCF), although new echinocandin agents are undergoing development and clinical trials such as rezafungin (Bader *et al*., 2018). These agents are semi-synthetic acylated cyclic hexapeptides, which demonstrate fungicidal activity by non-competitively inhibiting β-1, 3-glucan synthase, an enzyme that has an essential role in the construction of fungal cell wall components. Inhibition of this enzyme results in the absence of β-glucan in the cell wall in turn leading to a loss of structural integrity and ultimately cell death (Richardson and Warnock, 2003). As reviewed by Chang, Slavin and Chen (2017) the increased use of echinocandin

agents for the treatment of candidaemia has been driven by the fact that the class as a whole are generally well tolerated by patients, demonstrating few adverse reactions and low numbers of drug-drug interactions. This is a direct contrast with some azole agents such as voriconazole (Ruiz *et al*., 2019), or polyene agents like amphotericin B (Hamill, 2013). Some reports have suggested that 60% of patients with candidaemia will receive an echinocandin during the period of their acute treatment (Cleveland *et al*., 2012). However, the echinocandin agents are often difficult to administer due to a lack of oral formulations and a somewhat limited spectrum of action, although this is not of general concern regarding the treatment of candidaemia (Neoh, *et al*., 2018; Pappas *et al*., 2016; Eschenauer *et al*., 2014; Scott, 2012). Despite the evidence suggesting that an echinocandin agent may offer a superior treatment option to other antifungal agents (Lin *et al*., 2018; Eschenauer *et al*., 2013; Andes *et al*., 2012), it is compelling that Lausch *et al*. (2018) was able to demonstrate that even the introduction of a nationwide recommendation to use echinocandin agents as a first line option for candidaemia in Denmark, resulted in a low level of compliance from clinicians when compared to azole antifungal usage. Where an echinocandin had been used, Lausch *et al*. (2018) found a direct correlation in respect to mortality rates, and this was directly attributed to the patients receiving a more effective treatment in a timely manner.

First documented by Park (2005), resistance to the echinocandin class of antifungal agents still remains relatively low, with some estimates from the United States suggesting <3% resistance demonstrated in *Candida albicans* and most other *Candida* species (Castanheira *et al*., 2010), with the notable exception of *C. glabrata*, in which resistance appears to be increasing. The SENTRY antimicrobial surveillance programme for *C. glabrata* isolates, from 2006 to 2010, reported echinocandin resistance rates for *C. glabrata* between 8.0-9.3% (Pfaller *et al*., 2012b; Pfaller *et al*.,

2011b). This raised grave concerns for empiric therapeutic choices due to the already existent presence of triazole resistance, often cross-agent, demonstrated by *C. glabrata* (Pham *et al*., 2014b; Pfaller *et al*., 2012a; Pfaller *et al*., 2012b; Pfaller *et al*., 2008). Indeed, in the study by Pham *et al*. (2014b) nearly 36% of echinocandin resistant isolates of *C. glabrata* also exhibited resistance to fluconazole. In an epidemiological study within the United States of America, Grossman, Chiller and Lockhart (2014) demonstrated that the proportion of echinocandin resistant isolates in a single institution could be as high as 13.5%.

One particular mechanism for echinocandin resistance has been attributed to so-called hot spot mutations within the *FKS* gene region. First described in *Candida albicans*, this gene region has been shown to have a role in encoding a large integral membrane protein suspected to be 1,3- β-D-glucan synthase (Douglas *et al*., 1997). The *FKS* gene region includes three genes, *FKS1*, *FKS2* and *FKS3* and amino acid substitutions in these genes has been documented as the reason for resistance to treatment with echinocandin antifungal agents (Suwunnakorn *et al*., 2018; Alexander *et al*., 2013; Cleary *et al*., 2008; Douglas *et al*., 1994).

Distinct mutations within *FKS1* have proven to be responsible for resistance to all currently available echinocandin antifungal agents in isolates of *C. albicans* (Pham *et al*., 2014b; Katiyar *et al*., 2012; Pfaller *et al*., 2012b; Arendrup *et al*., 2010; Zimbeck *et al*., 2010), and mutations in both *FKS1* and *FKS2* have been associated with resistance in *C. glabrata* (Katiyar *et al*., 2012; Singh-Babak *et al*., 2012). Grossman, Chiller and Lockhart (2014) directly correlated the existence of mutations due to specific amino acid substitutions in the Fksp subunit of the 1,3- β-D-glucan synthase protein, with isolates of *C. glabrata* isolated from BSIs of patients who had failed echinocandin therapy.

Despite the obvious advantages of genetic alteration in response to antifungal pressure, such changes may often incur additional disadvantages in terms of continual survival or propagation (Borghi *et al*., 2014; Vincent *et al*., 2013; Clancy and Nguyen, 2011; Cowen, Kohn and Anderson, 2001). *FKS* mutation in particular has been shown to result in the generation of strains of *C. albicans* with thickened, chitin-rich cell walls and impaired ability for filament formation (Ben-Ami and Kontoyiannis, 2012).

1.4 Diagnostic testing in the clinical mycology laboratory

A large meta-analysis by Buehler *et al*. (2016) demonstrated that an approach, which included rapid molecular testing coupled with direct communication between laboratory and attending clinician, to confirm the identity of the microorganism causing the BSI, showed a significant reduction in mortality from BSIs. Whilst Buehler *et al*. (2016) made no formal recommendation as to how this identification should be achieved, it was noted that the potential to improve the time required to initiate targeted therapy was facilitated by the introduction of rapid identification techniques.

Microbial diagnostics in the clinical laboratory play a crucial role in assisting the selection of appropriate treatment regimes. This is not only from the perspective of the agent most likely to be effective against the infective organism, but also in reducing the use of ineffectual and unnecessary treatments. Such information helps both to reduce financial burden on healthcare providers and to maintain an effective arsenal of agents for treatment selection (Perez *et al*., 2013). Clinical laboratory diagnostics at their most basic level can be a simple classification of organism such as bacterium, virus or fungus to the complexity of genotyping specific isolates or subspecies. However, the most critical tool available is the *in vitro* testing of susceptibility to antimicrobial agents for each infective organism isolated from any given patient. There are many tools at the

disposal of the clinical diagnostic laboratory to achieve this, from systems that are labour-intensive such as micro or macro-broth dilution, to automated, commercial platforms such as BioMérieux's Vitek 2 (Alfouzan *et al*., 2017). It is essential that clinical diagnostic laboratories continue to develop the methodologies used to provide the data that actively inform clinical decisions. The introduction of new technology to microbial diagnostics such as matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) and more recently whole genome sequencing (WGS) are helping to lessen the manual manipulation required for the isolation, identification and characterisation of microbial isolates. In addition, these technologies often help to reduce the length of time required from isolation of causative organism of infection, to the selection of correct treatment, and infective resolution (Luethy *et al*., 2019; Nilsson *et al*., 2019; Vatanshenassan *et al*., 2018; Biswas *et al*., 2017a; Biswas *et al*., 2017b; O'Grady, 2016; McCann *et al*., 2015; Tran *et al*., 2015; Vogne *et al*., 2014).

The rapid detection of existing or emergent resistance before or during treatment with antifungal agents is a vital developmental tool allowing targeted effective therapy. This will form the underlying basis of many antifungal stewardship programmes in the era of broad-spectrum antimicrobial resistance (Perez *et al*., 2013). Cases of candidaemia occurring during therapy, sometimes known as breakthrough cases, are increasingly being reported (Astvad *et al*., 2018; Goemaere *et al*., 2018; Xiao *et al*., 2018; Berkow and Lockhart, 2017; Bizerra *et al*., 2014) and this is of importance as the landscape surrounding empirical antifungal selection changes. For example, the increase in recommendations for the use of echinocandin antifungal agents as first line treatment options instead of the triazole, fluconazole (Lausch *et al.*, 2018; Deorukhkar and Saini, 2016; Pappas *et al*., 2016; Eschenauer, Nguyen and Clancy, 2015), and the use of

echinocandin agents as prophylactic coverage (Enoch *et al*., 2018; Chang, Slavin and Chen, 2017; Bizerra *et al*., 2014; Alexander *et al*., 2013).

Conventional susceptibility testing in the laboratory is used to determine either the relationship of an agent compared to a breakpoint or the minimum inhibitory concentration (MIC) of any antimicrobial agent. In this context, the MIC is defined as the concentration of antifungal agent at which active growth is inhibited. It is possible for the point of no growth to be used as a determination of the concentration at which agents with fungicidal properties kill the fungus. This is often referred to as the minimum fungicidal concentration (MFC). Although, proof of cell death requires further viability studies and is rarely indicated or performed in the routine clinical laboratory (Fraser *et al*., 2007). The MIC value as read gives an indication of the most effective agent or agents for treatment of infection (CLSI, 2008). As previously discussed, methods of testing are often both time and labour consuming which can result in poor or ineffective empirical treatment selection (Pfaller *et al*., 2014a) and previous research has demonstrated patterns of antifungal activity that can vary between, and even within, species. For example, fluconazole and voriconazole appear to have a fungicidal effect on some isolates of *Candida parapsilosis*, but only produce a fungistatic response in others, thereby inhibiting fungal growth (Fraser, 2007). Voriconazole demonstrates good fungistatic activity against isolates of *Pichia kudriavzevii* (*Candida krusei*), which is considered intrinsically resistant to fluconazole; however, the fungicidal activity observed is not uniform and once again isolate specific variation is noted (Fraser, 2007).

Isolate and species-specific differences highlight the clinical need for fast and accurate susceptibility testing that has the ability to detect individual patterns of response to antifungal treatment regimes. This is essential for the effectiveness of therapy and is

therefore critical to treatment outcomes and on-going management of infection (CLSI, 2017b).

1.5 Genomic detection of resistance markers in *C. glabrata*

In the last decade novel genomic and proteomic technologies such as Pyrosequencing® and MALDI-TOF MS have revolutionised the speed and accuracy of identification of clinical isolates of fungi in the diagnostic laboratory (Fraser *et al*., 2016; Gorton *et al*., 2014; Borman *et al*., 2012). Pyrosequencing technology (originally designed by Pyrosequencing AB, now Biotage, Uppsala, Sweden) is a rapid DNA sequencing method that uses a novel enzymatic chemistry to sequence short (<70-bp) target regions of the nuclear rRNA gene cassette and is often considered as low-medium throughput sequencing most useful for small scale screening in the era of next generation sequencing (NGS) and whole genome sequencing (WGS) (King and Marsh, 2013). Even so, pyrosequencing has shown utility across most bioscience disciplines from the analysis of oral microbiome profiles (Ahn *et al*., 2011) and the species level identification of *Enterococcus* sp. (Zaheer *et al*., 2012) in bacteriology to the detection of single nucleotide polymorphisms (SNPs) in human genome analysis and tumour mutation detection (King and Marsh, 2013; Sahnane et al., 2013; Barbazuk and Schnable, 2011; Lavebratt *et al*., 2004). In *Candida* species, the D1-D2 portion of the 28S large rRNA gene (D1D2) and the internal transcribed region 2 (ITS2) have demonstrated particular utility in the identification of clinically relevant isolates (Borman *et al*., 2010; Borman *et al*., 2008; Linton *et al*., 2007). The application of the sequencing of these regions to the pyrosequencing platform enabled the accurate identification of 98% of 40 different species of clinically important yeast in less than 4

hours in the diagnostic laboratory (Borman *et al*., 2010). Previously unambiguous identification of the most frequently encountered agents of infection required a minimum of 24-48 hours and relied upon the colonial morphology and microscopy, and the biochemical assimilation of sugars in commercially available platforms such as AUXACOLOR 2 (BioRad, Marnes-la-Coquette, France) or API 20C (BioMérieux, Marcy-l'Etoile, France), often coupled with the knowledge of specialist microbiologists (Borman *et al*., 2012; Campbell *et al*., 1999).

The pyrosequencing process involves the primary polymerase chain reaction (PCR) amplification of target region DNA using biotinylated forward primers. Purification of the PCR product occurs using streptavidin-coated sepharose beads and a semi-automated washing system to denature double stranded DNA and remove non-biotinylated DNA strands. The streptavidin-bound biotinylated single stranded DNA is eluted into a reaction plate containing a mixture of annealing buffers and specific primers and the combined mixture is subjected to automated sequencing which includes the ordered addition of enzymes, substrate and deoxynucleotide triphosphates (dNTPs). The incorporation of specific dNTPs into an extending complementary non-biotinylated DNA strand is detected via the production of inorganic pyrophosphate, which is released and converted to adenosine triphosophate (ATP). Generated ATP drives a luminescent reaction due to the presence of the enzymes sulfurylase and luciferase. Unincorporated nucleotides/ATP are removed by the enzyme, apyrase before the introduction of the next nucleotide into the sequence. Each visible light signal generated is proportional to the number of nucleotides incorporated into the analysed DNA strand (Gharizadeh *et al*., 2006; Ronaghi and Elahi, 2002; Ronaghi, 2001; Ronaghi, Uhlén and Nyrén, 1998).

Rapid PCR based methodologies such as pyrosequencing, asymmetric PCR and multiplex systems have been employed to determine the presence of *FKS1* and/or *FKS2* mutants (Zhao *et al*., 2016; Dudiuk *et al*., 2014; Pham *et al*., 2014a). It has been noted that the position and number of mutations in *FKS* selectively influences *in vitro* and *in vivo* susceptibilities to the echinocandin class of antifungal agents (Pham *et al*., 2014b). Mutations within these genes result in a conformational change in the target for the echinocandin antifungal agents, 1, 3-D-glucan synthase (Lackner *et al*., 2014) and therefore may also be detectable using a proteomic approach like MALDI-TOF MS as a rapid identifier of antifungal resistance (Delavy *et al*., 2019; Paul *et al*., 2018; Vatanshenassan *et al*., 2018; Vella *et al*., 2017; De Carolis *et al*., 2012; Kelly and Kavanagh, 2010).

1.6 Detection of proteins for the identification and classification of microorganisms

Since the early work of O'Farrell (1975) and Klose (1975), the analysis of proteins within biological systems has been refined and adapted from a simplistic comparison between individual cultures of *Escherichia coli*, to the analysis of whole mammalian cell processes and even host-pathogen interactions (Beltran *et al*., 2017; Lum and Cristea, 2016; Schmidt *et al*., 2010). The study of the whole set of proteins within a biological system or organism in this way is termed proteomics and is considered a core component to the basic analysis of cell function, having been developed into an essential tool in the understanding of many biological processes.

The detection and quantification of proteins from biological samples has been undertaken using various iterations of gel electrophoresis for example, 2-dimensional

polyacrylamide gel electrophoresis (2D-PAGE), to separate, identify and characterise proteins within a given biological system (Fey *et al*., 1997). Microbial proteomics has advanced from the characterisation of single organisms undergoing a specific physiological process, i.e. stress/starvation (Bar *et al*., 2007), or at a specific point in time (Schmidt *et al*., 2010) to the presentation of highly complex systems such as host-pathogen interactions (Beltran *et al.*, 2017) and mixed microbial communities (Marlow *et al.*, 2016; Siggins *et al*., 2012). The additional application of other developing biochemical techniques to elements of proteomics has further enhanced the specificity of 2D-PAGE and increased its utility in the understanding of the roles of proteins in biological systems, particularly in the clinical setting of infection. Techniques have included the use of pH gradients and variable forms of protein labelling such as fluorescence or isotopic labelling, as reviewed by Pérez-Llarena and Bou (2016).

The continual development of electrophoresis-based proteomic techniques has recently seen a shift towards a "bottom-up" or "shot gun" approach to protein detection (Zhang *et al*., 2013; Washburn *et al*., 2001). This has been aided by the inclusion of sophisticated mass spectrometry techniques, which has shifted the focus of proteomic investigation from whole proteins, to singular or small clusters of proteolytic peptides (Boulund *et al.*, 2017; Otto, Becher and Schmidt, 2014; Duncan, Aebersold and Caprioli, 2010; Aebersold and Mann, 2003). "Bottom-up" proteomics involves the digestion of intact proteins, into small fragments and their subsequent analysis to provide a picture of the whole proteome at the same time, a direct contrast to a more conventional "top-down" approach whereby intact, whole proteins are analysed for function. The main advantage of the "bottom up" approach is its ability to enable the handling of large molecular weight proteins because they are broken into manageable fragments (Zhang *et al*., 2013). However, there is an element of intrinsic uncertainty in

this approach to proteomics due to the potential limitations of predicting whole proteins from only a collection of identified peptides, and ambiguities which surround the origin of redundant or nonsense protein sequences (Yates, Ruse and Nakorchevsky, 2009). It is now common to use a mixture of both old and new approaches, a so-called "middle-down" approach which allows the analysis of large proteins as fragments, but also helps to reduce the redundancy present in the data generated (Zhang *et al*., 2013). The improved sensitivity of mass spectrometers, in combination with advances in sample preparation and protein fractionation technologies, has broadened the study of the whole set of proteins within a biological system (Pérez-Llarena and Bou, 2016). The use of liquid chromatography with the addition of a mass spectrometer detector and analyser (LC-MS/MS) in clinical biochemistry for the detection of substances of abuse, therapeutic agents and the rudimentary detection of metabolic compounds has become routine (Dias *et al*., 2016; Decosterd *et al*., 2010; Miller *et al*., 2008). Given that the majority of clinical diagnostic laboratories no longer have the capabilities to undertake intensive, time consuming, and highly skilled gel-based proteomic work, there is value in adopting and developing cross discipline technologies. At the same time, there is evermore pressure to automate and standardise techniques in the clinical diagnostic laboratory (Kothari *et al*., 2014; Bourbeau and Ledeboer, 2013). However, the value of proteomics to clinical practice remains abundantly apparent and the evolution of laboratory techniques is paramount to the effectiveness of any diagnostic service (Pérez-Llarena and Bou, 2016; Otto, Becher and Schmidt, 2014). Proteomic analysis of fluconazole resistant isolates of *C. albicans* has demonstrated metabolic shifts related to cellular and membrane protein changes (Yan *et al*., 2007) and this has been replicated in *C. glabrata* (Yoo et al., 2012). Several studies have also presented data indicating that cell wall alterations, at least in *C. albicans*, have a key role in the interaction of the

echinocandin agents and this yeast (Rueda, Cuenca-Estrella and Zaragoza, 2014; Vavala *et al*., 2013).

1.7 MALDI-TOF MS and the identification of fungi

MALDI-TOF MS utilises the ablation power of a laser to ionise metabolic proteins within biological samples and provide a fragmented proteomic fingerprint of an organism. This methodology was first described as a potential tool in diagnostic microbiology by Claydon *et al*. (1996). Subsequently, the rapid development in the understanding of relationships in peptide and protein analysis (Lewis, Wei and Siuzdak, 2000) and the accessibility of commercial platforms over the past decade, has driven a revolution that has seen MALDI-TOF MS for the identification of bacteria and fungi as agents of infection replacing many biochemical and morphological laboratory techniques. Many clinical diagnostic microbiology laboratories incorporate some form of mycology identification as part of their remit, and the introduction of MALDI-TOF MS has extended the capabilities of some diagnostic microbiology laboratories especially in terms of yeast identification. There is a large amount of evidence to suggest that the use of MALDI-TOF MS in the identification and classification of fungi is highly successful and provides some aid to an ever-diminishing pool of expertise in clinical mycology within the diagnostic clinical laboratory (Borman *et al*., 2019; Luethy *et al*., 2019; Borman *et al*., 2018b; Hou *et al*., 2018; Angeletti, 2017; Fagerquist, 2017; Fraser *et al*., 2017; Borman *et al*., 2017; Fraser *et al*., 2016; Fatania *et al*., 2015; Gorton *et al*., 2014; Vyzantiadis, Johnson and Kibbler, 2012; Aebersold and Mann, 2003). For those laboratories with rudimentary or limited knowledge in mycological identification, national reference centres such as the PHE MRL, where specialised

testing is performed, are able to aid in the confirmation, identification and classification of organisms (Borman *et al*., 2012). It is the experience of the author that despite the widespread introduction and ease of use of rapid identification technologies such as MALDI-TOF MS, mycology reference centres continue to provide a significant and expanding service for many clinical diagnostic microbiology laboratories.

However, to date, there has been very limited success with the expansion of MALDI-TOF MS into the detection of antifungal resistance. Undoubtedly this is a critical area for research given not only the expanding number of fungal infections and potential agents of disease, but also the limitations surrounding the development of novel antimicrobial targets and compounds (Vatanshenassan *et al*., 2018; De Carolis *et al*., 2012; Marinach *et al*., 2009). Research studies have demonstrated that under specific conditions it is possible to provide a qualitative estimate of an organism's susceptibility profile using MALDI-TOF MS, but none have demonstrated a single tool that could be used in the routine throughput setting of clinical diagnostics where time and money are both short, without the need for an individual set up or system to be in place (Delavy *et al*., 2019; Dortet *et al*., 2018; Vogne *et al*., 2014; De Carolis *et al*., 2012).

It has been demonstrated that changes in relative protein abundance seem to correlate with resistance gene expression for many proteins, and this may be used to demonstrate an effect in response to the presence of antifungal agents from all classes of agents (Saracli *et al*., 2015; Marinach *et al*., 2009).

At the most basic level, if the current diagnostic laboratory MALDI-TOF MS procedures could provide some guidance as to susceptibility of a clinical isolate at the same time as performing the identification of an organism, this would provide a simple and desirable outcome for management of therapeutic decisions.

The MALDI-TOF MS identification system uses an in-built, often commercially supported, database to compare known protein derived spectral profiles to unknown sample spectral profiles, to enable the identification of an organism coupled with a confidence value. Databases have proven to be complex and often difficult things to consolidate, replicate and disseminate, with many centres providing their own methodology and consequently developing specific databases from their own collections (Borman *et al*., 2019; Borman *et al*., 2017; Fraser *et al*., 2017, Normand *et al*., 2017a; Lau *et al.*, 2013). There is currently no consensus regarding methodology and there are a number of commercially available databases as well as independent institution-led databases. Different groups have demonstrated that the spectral profiles created differ greatly dependent upon the method of protein extraction and the spread and variety of organisms included (Normand *et al*., 2017a; Fraser *et al*., 2016; Gorton *et al*., 2014; Lau *et al*., 2013). This has led to many research groups limiting their advances to only one methodological approach or to others restricting developments to one species complex or group in order to expand a specific area of interest (Borman *et al*., 2019; Borman *et al*., 2017; Fraser *et al*., 2017, Normand *et al*., 2017a). There is currently no one database that has a broad coverage of all clinically relevant microorganisms. It would seem that given the amount of work already undertaken on individual databases it is unlikely a single platform to suit all will be found. Recently, the use of an online database for the retrospective analysis of spectral profiles has proven a useful tool (Normand *et al*., 2017a; Normand *et al*., 2017b), but once again the largest caveat of a database still holds, it is limited to methodology, content and curation.

The PHE MRL currently uses a commercially available database (Bruker Daltonics GmBH, Bremen, Germany), alongside a web application (Normand *et al*., 2017a) and

an internally created database of unusual, or geographically distinctive, profiles to provide a broad coverage of identification of fungal pathogens (Fraser *et al.*, 2016). As such, the first step to ascertaining if spectral differences exist between susceptible and resistant isolates of *C. glabrata* would be to generate profiles for them. Commercially available databases already cover a wide variety of spectral fingerprints seen in *C. glabrata*, so the MRL internally curated database (MRLDB) constructed as part of on-going diagnostic development, is ideally placed to create specific profiles (main spectrum profile; MSP) for resistant isolates.

1.8 Study aim

The focus of the work undertaken has been driven by a need to improve clinical services in diagnostics, with the aim of better informing clinical management decisions quickly and accurately thereby effecting direct patient impact (Borman *et al*., 2018b; Lin *et al*, 2018; Xiao *et al*., 2018; Lockhart *et al*., 2017; Eschenauer *et al*., 2014; Alexander *et al*., 2013). All efforts to use techniques that are readily available to most high performing clinical diagnostic laboratories has been made. Most well-equipped clinical microbiology laboratories should be able to access a biologically appropriate mass spectrometry system capable of adaptation to the detection and analysis of microbial proteins. This may often be achieved by the establishment of a close working relationship with a clinical biochemistry laboratory, and in so doing provides a good example of cross-discipline integration in biomedical science, a topic which is becoming more relevant to the development of clinical diagnostics (European Commission, 2013).

By using a methodical approach, *C. glabrata* and the echinocandin class of antifungal agents were used as a laboratory model of antifungal resistance to determine the

viability of a laboratory bench-top genomic platform; pyrosequencing, and the proteomic platforms; LC-MS/MS and MALDI-TOF MS for the detection of resistance. Based on this, the viability of MALDI-TOF MS which is in common usage in clinical microbiology laboratories throughout the UK for the identification of fungi (Borman *et al*., 2019; Borman *et al*., 2018b; Fraser *et al*., 2017; Borman *et al*., 2017; Fraser *et al*., 2016; Fatania *et al*., 2015; Gorton *et al*., 2014) was assessed for the detection of resistance profiles in *C. glabrata*.

The overarching aim was to enable the rapid detection of specific antifungal resistance markers within a clinically effective time frame in the diagnostic laboratory, which would in practice, have the potential to positively influence therapeutic decisions and ultimately treatment outcomes.

1.8.1 Study objectives

To enable achievement of the study aim, a series of objectives were identified during the project development phase:

Objective 1: To enhance the existing pyrosequencing-based method to incorporate the detection of point mutations in the *FKS* gene that may indicate resistance to echinocandin antifungal agents in *C. glabrata*.

> ***Objective 1.1*:** Compile paired panel of *C. glabrata* exhibiting susceptible and resistant characteristics.

Objective 2: Attempt to visualise and characterise proteins using LC-MS/MS and MALDI-TOF MS that may be increased or decreased in relative abundance in isolates identified as containing genetic mutations indicative of resistance.

Objective 3: Using MALDI-TOF MS, generate peptide mass profiles for echinocandin resistant and susceptible isolates and determine if any identified proteins can be distinguished within them.

> ***Objective 3.1*:** To develop or create whole cell protein extraction techniques which will maximise recovery of cell wall and cell membrane components.

> ***Objective 3.2*:** To explore the operational parameters of MALDI-TOF MS in the clinical laboratory to enhance protocols and enable specific identified protein detection.

Objective 4: To create a robust database for the detection of pre-existing or inducible resistance markers by MALDI-TOF MS in wild type clinical isolates of *C. glabrata*.

2. MATERIALS AND METHODS

2.1 Collection/Selection of *C. glabrata* isolates

Isolates of *C. glabrata* from the National Collection of Pathogenic Fungi (NCPF) were revived from storage in liquid nitrogen (LN2). Isolates were selected based upon previous antifungal resistance testing, where resistance to the echinocandin class of antifungal agents had been demonstrated. This had been determined by microbroth dilution testing against CSP, the first echinocandin class of antifungal to be clinically available (Kartsonis, Nielsen and Douglas, 2003). Storage vials were removed from LN2 storage and placed at -80°C in a container of 100% propanol to reduce the cryothermic destruction of cells. After a period of 24 hours, the vials were removed to room temperature to fully thaw. An aliquot was spread onto Sabouraud dextrose agar containing 100 mg/L chloramphenicol (SABC; PO0161A; Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 30°C for 48 hours. Once revived, isolates were passaged at least twice, to ensure adequate log phase growth was achieved (Borman *et al*., 2006). When fully revived, all isolates were subjected to confirmatory echinocandin resistance testing by E-test as described by Arendrup *et al*. (2010) as detailed in Section 2.2.1.

Simultaneously between June 2015 and July 2018, all clinical isolates submitted to the Public Health England (PHE) Mycology Reference Laboratory (MRL) that demonstrated phenotypic resistance or raised MIC values to a single, or multiple echinocandin agents were catalogued and stored as described by Borman *et al*. (2006). Table 1 provides details about the isolates used throughout this work, including the original source and referral location of each isolate.

Table 1. Details for all isolates demonstrating echinocandin resistance included in this study. Table presents all isolates tested as part of this study alongside information about clinical origin and location of referral, if available (Fraser *et al.*, 2019a).

Key: N/A = not available, [a-g] = individual London healthcare centres.

Isolate	Source	Location
NCPF Number		
8714	N/A	N/A
8715	N/A	N/A
8745	N/A	N/A
8814	N/A	N/A
8919	Blood	N/A
Clinical Number		
61	Pus	Gloucester
67	Blood	Dublin, Ireland
68	Blood	London[a]
70	Blood	London[b]
71	Wound	London[c]
72	Abdominal Drain	Liverpool
73	Blood	London[c]
74	Perianal Swab	Sheffield
75	Blood	London[d]
76	Wound	London[d]
77	Bile Leak	London[e]
78	Blood	London[f]
79	Blood	Liverpool
80	Peritoneal Fluid	London[g]
81	Nephrostomy Urine	London[d]

2.2 Susceptibility testing of *C. glabrata* isolates

2.2.1. Epsilometer (E-test) susceptibility testing

A suspension of *C. glabrata* was prepared in 4 mL normal saline (0.9% sodium chloride) to a cell density of approximately 2.5 x 10^6 CFU/mL, as determined by monitoring percentage transmission at an absorbance of 530 nm using a spectrophotometer (Jenway 6305, Jenway, USA). An RPMI agar plate (BioMérieux, Marcy-L'Etoile, France; AEB122180) was inoculated with the prepared cell suspension and allowed to dry. Plastic strips (E-tests) coated on one side with a concentration gradient of antifungal agent (BioMérieux, Marcy-L'Etoile, France; caspofungin, product code 532418; anidulafungin, product code 532000; micafungin, product code 535708) were placed onto the inoculated RPMI agar plate to perform rapid susceptibility testing as previously described (Arendrup *et al*., 2010). The plate was incubated at 35°C (±2°C) for 48 hours, with MIC values being read at both 24 and 48 hours. All testing of MCF in this study was performed by this method only.

2.2.2. Microbroth dilution susceptibility testing

Microbroth dilution susceptibility testing was performed according to the Clinical Laboratory Standards Institute document M27-A3 (CLSI, 2008). Suspensions of CSP and ANF were prepared to an initial working solution of 10,000 μg/mL. Thus 10 mg anidulafungin powder (Pfizer Inc., USA, product code PF3910960) was dissolved in 1 mL dimethyl sulphoxide (DMSO; Sigma Aldrich, USA, product code 472301), and 10.3 mg caspofungin diacetate (Sigma Aldrich, USA, product code SML0425; activity

of powder = 97%) was dissolved in sterile distilled water. Concentration gradients were achieved by serial doubling dilutions in RPMI liquid media (RPMI-1640 with L-glutamine and sodium bicarbonate; Sigma Aldrich, USA, product code R8758) supplemented with 2% glucose (Sigma Aldrich, USA, product code G8270) and buffered to pH 7.0 ± 0.1 at 25°C ± 0.1 with a 0.165 M solution of 4-morpholinepropanesulphonic acid (MOPS; Sigma Aldrich, USA, product code M1254), to a test concentration range of 0.015 μg/mL to 16 μg/mL. Each concentration was added to a column of wells in a sterile 96- round well microtitre plate (Corning Inc., USA; BC013) in volumes of 100 μL.

Test organisms were freshly sub-cultured onto SABC agar for 24 hours prior to testing. A suspension with an approximate cell count of between 1-5 x 10^6 CFU/mL was prepared in normal saline (0.9%; w/v NaCl) by monitoring percentage transmission on a spectrophotometer as previously described (Section 2.2.1). A volume of 0.1 mL yeast/saline suspension was added to 4.9 mL RPMI, then 0.5 mL of this diluted suspension was added to 9.5 mL RPMI resulting in a working volume of twice the 1000-fold final testing dilution of approximately 2.5 x 10^3 CFU/mL. An aliquot of 100 μL RPMI diluted yeast suspension was added to each well of the prepared microtitre plates and each plate was incubated at 35°C ± 2°C for 48 hours.

After this time, minimum inhibitory concentration (MIC) values were read visually with the aid of a reading mirror. Growth in each well of the concentration gradient was compared to a growth control well, which did not contain antifungal agent and a prominent reduction in turbidity within a well (generally >50%) is the value at which an echinocandin agent is considered to exert a significant effect upon the organism being tested (Pfaller *et al*., 2014a). A negative control well containing no agent or organism was also included to ensure sterility of microtitre plate and media.

2.2.3 Macro-broth dilution susceptibility testing

Large volume (macro-broth) susceptibility testing was undertaken in an attempt to increase the biomass available for protein extraction at an earlier time interval than 48 hours. As previously described in Section 2.2.1, a yeast cell suspension with an approximate cell count of between 1-5 $x10^6$ CFU/mL was prepared and 100 μL of this was added to 9900 μL RPMI liquid medium to provide an approximate cell density of between 1-5 $x10^4$ CFU/mL. A test suspension of 2 mL was prepared by adding 1 mL of the inoculated growth media to 1 mL of the pre-prepared concentration of antifungal in a 10 mL glass tube (International Scientific Supplies Ltd (ISS), Silchester, UK, product code GBT021). This working solution was placed on a fixed speed rotating mixer revolving at 20 rpm (Stuart fixed speed rotator SB2, carl Roth GmBH & Co. KG) at 35°C for 24-48 hours. Sample volumes of 100 μL were removed at periodic time intervals and subjected to protein extraction as described in Section 2.5.

2.2.4 Interpretation of susceptibility testing results

In clinical testing, there is an allowance of two doubling dilutions difference between testing and different operators to allow for variations in media, temperature, inoculation and interpretation (Eschenauer *et al.*, 2014; Pfaller *et al*., 2014a; Pfaller *et al*., 2011b). This protocol was applied to all susceptibility testing within this study and if a result caused a discrepancy outside of this range, or the dilution reported changed the overall classification of an isolate, testing was repeated. The MIC breakpoints for susceptibility of *C. glabrata* to the echinocandin class of antifungal agents are fully described by

CLSI (2017a; 2017b). As a reference, the values used throughout this study are displayed in Table 2.

Table 2. Echinocandin breakpoint values for the interpretation of susceptibility in *C. glabrata* by microbroth dilution as recommended by CLSI. This table shows the interpretative breakpoints in μg/mL for the classification of echinocandin susceptibility in *C. glabrata* used throughout the research undertaken as part of this study (CLSI, 2017a; CLSI, 2017b).

Caspofungin (CSP) / Anidulafungin (ANF)		
	≤0.125 μg/mL	Susceptible
	0.25 μg/mL	Intermediate
	≥0.5 μg/mL	Resistant
Micafungin (MCF)		
	≤0.06 μg/mL	Susceptible
	0.125 μg/mL	Intermediate
	≥0.25 μg/mL	Resistant

Internal quality control (IQC) isolates were included for each test to assess the validity of each prepared drug batch and test conditions. *C. parapsilosis* NCPF 8334 (ATCC 22019) and *P. kudriavzevii* (*C. krusei*) NCPF 3953 (ATCC 6258) were the isolates used as per standard practice (Pfaller *et al*., 2011a; CLSI, 2008). IQC MIC values were expected to fall within the following ranges:

CSP/ANF/MCF	NCPF 8334	≤ 2 μg/mL
	NCPF 3953	≤ 0.25 μg/mL

In all tests, the MICs of the IQC isolates were within accepted limits (data not shown).

2.2.5 *In vitro* induction of resistance

As Bordallo-Cardona *et al*. (2017) reported that it was possible to promote echinocandin resistance by inducing *FKS2* mutations *in vitro* by exposure to increasing concentrations of MCF, an attempt was made to induce resistance using the E-test method of susceptibility testing and NCPF isolates 8714, 8715 and 8745, all of which had shown echinocandin susceptibility upon revival from long-term storage.

As previously described (Section 2.2.1) cell suspensions were prepared in normal saline and used to inoculate RPMI plates for E-test. After 72 hours exposure, a sub-culture of the test isolate from nearest the MIC value, or of any macro-colonies from within the zone of inhibition, was taken onto a fresh SABC agar plate and testing was repeated. An inoculum was also sub-cultured onto a SABC agar slope for long-term storage. This process was repeated with each NCPF isolate that had previously demonstrated susceptibility to the echinocandin antifungal agents for up to a total of six passages. The presence of macro-colonies within areas of inhibition was taken as indicative of resistant sub-populations and these macro-colonies were stored, re-tested and further sub-cultured. All macro-colonies were subjected to pyrosequencing analysis to determine *FKS* sequences as described in Section 2.3.2.

The first series of experiments used CSP, but all subsequent experimental passages were tested against ANF, which has been shown to be a better indicator of resistance *in vitro* across the whole group of echinocandin class antifungal agents (Pfaller *et al*., 2014c; Espinel-Ingroff *et al*., 2013; Shields *et al*., 2013).

2.3 Genomic detection of echinocandin resistance

2.3.1 Primer design for amplification of *FKS* mutations

A region of the *C. glabrata* open reading frame was selected for sequence analysis based on the position of *FKS* mutations which have been shown to confer echinocandin resistance *in vitro* for *Saccharomyces cerevisiae* (Douglas *et al*., 1994), *C. albicans* (Park *et al*., 2005) and *C. glabrata* (Dudiuk *et al*., 2014).

The forward and reverse primers used for PCR were based on *C. glabrata FKS*1 (CgFKS1), GenBank accession no. KF211456.1 (Bizerra *et al*., 2014) and for *C. glabrata FKS*2 (CgFKS2) primers were based upon GenBank accession no. HM366442.1 (Niimi *et al*., 2012). Primers were created at Invitrogen (Thermo Fisher Scientific, UK) and constituted as detailed in Table 3. Sanger sequencing was performed by GeneWiz (formerly Beckman Coulter Genomics, Takeley, UK) to assess the efficiency of the primer set prior to use.

Table 3. Pyrosequencing primers used for detection of *FKS*1 and *FKS*2 mutations. The full sequence of the *FKS1* and *FKS2* pyrosequencing primers that were constructed for this study (Fraser *et al*., 2019a).

	Forward	Reverse
FKS1	TTGTCTTACCTGGTTTGGGTTACT	GTACATCTCATGGTAGTGGTAGAC
FKS2	TTATCTTATTTAGTTTGGGTTACA	GTACATCTCATGGTAGTAGTTGAT

2.3.2 Pyrosequencing and rapid detection of *FKS* mutations

Pyrosequencing was performed as previously described for yeast by Borman *et al*. (2010) using PyroMark ID and PyroMark Q96 reagents (Qiagen, Gaithersburg, MD, USA; Product code: 972804). Biotinylated forward primers were generated for pyrosequencing using the primers designed as described in Section 2.3.1. The reverse primer (Cg*FKS*1/2 reverse) was used as the pyrosequencing analysis primer at a working concentration of 20 μM as analysis of the predicted location of hot spot mutations, and the limitations of pyrosequencing technology had previously suggested that this would maximise the chances of successful amplification (Gharizadeh *et al*., 2006). It was important to consider therefore that all the sequences generated by this system would be in reverse. When using automated online nucleotide alignment software such as BLASTn to determine the accuracy of the short sequence read, built in algorithms reverse and compliment the data (Altschul *et al*., 2001) so the use of the reverse primer was accounted for at this stage. However, to enable the real-time analysis of sequences generated by the pyrosequencing analysis software, IdentiFire®, the creation of a FASTA format file was necessary for each possible mutation combination within *FKS*1 and *FKS*2. The full FASTA file containing all predicted *FKS* sequences can be found in Appendix II. Tables 4 and 5 detail the regions of interest considered in the pyrosequencing sequences as outlined by Dudiuk *et al*. (2014) and Pfaller *et al*. (2011a).

Table 4. Sequence and codon positions of *C. glabrata FKS1* region. Sequence and codon position of *C. glabrata FKS1* region with highlighted hot spot mutation regions, the expected location of the reverse primer (RP) used for pyrosequencing and the expected sequence generated when read from right to left (Dudiuk *et al*., 2014; Pfaller *et al*., 2011a).

TAC	**TAC**	**TTC**	**TTG**	**ATT**	**CTA**	**TCT**	**CTA**	**AGA**	**GAT**
623	624	625	626	627	628	629	630	631	632
ATG	ATG	AAG	AAC	TAA	GAT	AGA	GAT	TCT	CTA
CCA	ATC	AGA	ATT	TTG	TCT	ACC	ACT	ACC	
633	634	635	636	637	638	639	640	641	
GGT	TAG	TCT	TAA	AA					

	RP
	Hotspot

	Pyro

Table 5. Sequence and codon positions of *C. glabrata FKS2* region. *FKS2* sequence and codon position of *C. glabrata* with highlighted hot spot mutation regions, the location of the reverse primer (RP) used for pyrosequencing and the expected sequence generated, when read from right to left (Dudiuk *et al*., 2014; Pfaller *et al*., 2011a).

TAC	**TTC**	**TTC**	**TTG**	**ATT**	**TTG**	**TCT**	**CTA**	**AGA**	**GAC**
657	658	659	660	661	662	663	664	665	666
ATG	AAG	AAG	AAC	TAA	AAC	AGA	GAT	TCT	CTG
CCT	ATC	AGA	ATT	TTA	TCA	ACT	ACT	AAC	
667	668	669	670	671	672	673	674	675	
GGA	TAG	TCT	TAA	AA					

	RP
	Hotspot

	Pyro

2.4 Detection of proteins to indicate resistance by LC-MS/MS

LC-MS/MS was performed on prepared extracts of *C. glabrata* isolates NCPF 8814 (echinocandin resistant, *FKS* mutation present) and NCPF 8745 (echinocandin susceptible) to compare and characterise proteins extracted from both isolates. This was performed at the proteomics facility of the University of Bristol. Two different protein extracts were compared: one of which was optimised for LC-MS/MS techniques (Extract A) and the other designed for MALDI-TOF MS identification (Extract B).

2.4.1 Preparation of samples for LC-MS/MS

Isolates NCPF 8814 and NCPF 8745 were sub-cultured as detailed in Section 2.1, following a period of incubation at 30°C for 48 hours, a suspension of yeast equivalent to 2 McFarland standard was prepared in a sterile plastic 1.5 mL micro-centrifuge tube with 300 µL de-ionised water (Honeywell, Muskegon, Michigan, USA; product code: 38796) followed by 800 µL of 100% ethanol (Sigma Aldrich, St. Louis, Missouri, USA; product code: 32205). The prepared samples were mixed using a vortex mixer for 30 seconds then centrifuged at 13,226 g for 3 minutes. All liquid supernatant was removed, the centrifugation step was repeated, and any residual liquid was removed (Fraser *et al*., 2016). At this stage the biomass pellets requiring LC- MS/MS analysis extraction (Extract A) were forwarded to the proteomics facility, University of Bristol. The protein purification methodology created for this project by the proteomics facility, University of Bristol, was developed from Yoo *et al*. (2012) and can be found in full in Appendix III. The parameters used for LC-MS/MS were as described by Goggs *et al*. (2013).

2.4.2 Extraction protocol for LC-MS/MS – MALDI-TOF MS analysis

Following the removal of supernatant after centrifugation as described in Section 2.4.1, biomass pellets were left to air dry, typically for between 3-5 minutes. Once dried, pellets were re-suspended in 70% (vol/vol) formic acid (Acros Organics, New Jersey, USA: product code: 147930250) and left at ambient temperature for 5 minutes. Protein precipitation was achieved by the addition of 100% acetonitrile (Honeywell, Muskegon, Michigan, USA; product code: 34967) to the re-suspended pellet and the solution was mixed using a vortex mixer for 30 seconds. Each sample was centrifuged at 13,226g for 3 minutes and the resulting supernatant was used for both LC-MS/MS analysis and MALDI-TOF MS analysis (Fraser *et al*., 2016). LC-MS/MS was performed once for each isolate. MALDI-TOF MS was performed in triplicate on at least 3 independent test runs.

2.4.3 LC-MS/MS data analysis

LC-MS/MS analysis resulted in mass spectrum profiles as spectral data files and the method for data acquisition can be found in Appendix III. Each mass spectrum generated by the analyser was compared against a species-specific protein database in forward and reverse, or false, formats (Boulund *et al*., 2017; Duncan, Aebersold and Caprioli, 2010). Each match of a spectrum to a peptide in these databases was given a score indicating how closely the spectrum matches the predicted peptide sequence. The false search was expected to generate low match scores, due to the expectation that the protein sequences generated should be nonsensical. The software analyses the distribution of the scores matched to both the genuine and false databases and calculates

a score cut off, where there is only a 5% chance that a resolved peptide matched the false database and is therefore not an accurate predicted peptide sequence, this generates a false discovery rate (FDR; Zhang *et al.*, 2011). All peptides with a score below this calculated cut-off are termed low confidence peptides. A high confidence level was set at 1%, equivalent to only 1 in every 100 peptides matching the false database. Therefore, at the 1% high confidence level it can be reasoned that there is 99% confidence that every peptide present in the extraction was a genuine peptide from a protein matched in the species-specific database. Spectral data was analysed using Protein Discoverer™ (Thermo Fisher Scientific Version 1.4, USA) and the UniProt *C. glabrata* isolate NCPF 3309 (ATCC 2001; [284593]) using the SEQUEST algorithm (Thermo Fisher Scientific, USA). Each mass spectra (raw data) generated by the analyser was searched against a species-specific protein database in forward and reverse (false) formats. Each match of a spectrum to a peptide in these databases is given a score indicating how closely the spectrum matches the predicted peptide sequence.

If a protein was found to be uncharacterised in the LC-MS/MS initial database search, then a subsequent search using the database for the most abundantly studied fungal organism, *Saccharomyces cerevisiae* and homologous proteins was used to predict likely protein candidates. Alternative searches were also conducted in a non-organism specific manner using UniProt, an online aggregator of several protein databases (UniProt Consortium, 2017).

2.5 Preparation and analysis of samples by MALDI-TOF MS

2.5.1 Basic protein extraction

Extraction of proteins was achieved using a methodology recommended by the MALDI-TOF MS manufacturer (Bruker Daltonic GmBH, Bremen, Germany) and optimised by users working in diagnostics in clinical mycology (Fraser *et al.*, 2016; Gorton *et al.*, 2014; Fatania *et al.*, 2014).

C. glabrata isolates were grown for 24 hours at 30°C on a SABC agar plate. Each isolate was tested in triplicate on at least 3 independent test runs. An amount of biomass, roughly equivalent to 1 μL was removed using a 1 μL plastic loop from the plate into a sterile plastic conical tube containing 300 μL of de-ionised water, 800 μL 100% ethanol was added, and the tube was then vortex mixed for 30 seconds. The mixture was centrifuged at 13,226 *g* for 3 minutes before the supernatant was removed and discarded. A repeat centrifugation step was performed to fully remove the ethanol/water mixture from the biomass pellet. The pellet was allowed to air dry for no more than 15 minutes, further allowing for ethanol evaporation. The pellet was re-suspended in a volume of 70% (vol/vol) formic acid equivalent to the size of the pellet, and in practice this ranged from 30-50 μL. The suspension was incubated at ambient temperature for 5 minutes before an equal volume of 100% acetonitrile was added and the tube was gently mixed. Following a further ambient incubation of no less than 5 minutes, the tube was mixed and centrifuged at 13,226 *g* for 3 minutes. The supernatant was then placed onto a 96-spot polished steel target plate (Bruker Daltonics GmBH, Bremen, Germany; product code: 8280800) in volumes of 1 μL. Once the supernatant had evaporated, 1 μL of α-Cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker

Daltonics GmBH, Bremen, Germany; product code: 8255344) reconstituted in 250 µL organic solvent (OS) comprised of 25 µL trifluoroacetic acid (TFA; Honeywell, Muskegon, Michigan, USA; product code: 302031); 475 µL deionised water and 500 µL 100% acetonitrile, was added to each spot and allowed to air dry as shown in Figure 1.

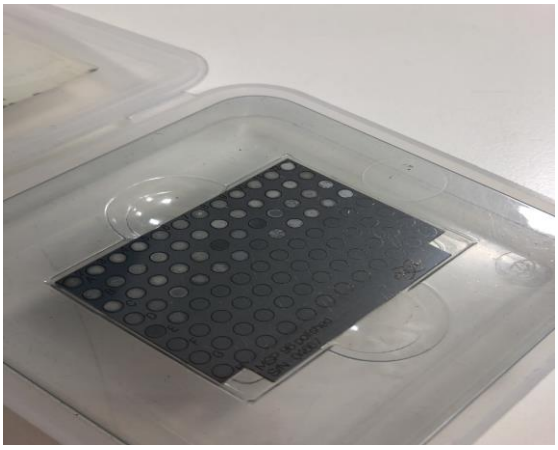

Figure 1. <u>A prepared MALDI-TOF MS target plate.</u> A stainless-steel target plate for MALDI-TOF MS analysis with matrix coated air-dried samples (author's own image)

2.5.2 Extraction of proteins after antifungal exposure

For those experiments involving the exposure of isolates to antifungal agents, a series of cell wash steps were introduced prior to protein extraction protocol as laid out in Section 2.5.1. The wash steps were designed to help the removal of residual growth media and antifungal carry-over (Nguyen *et al*., 2009), which may cause discrepancies in the final spectral acquisition. This process involved the removal of 100 µL growth media from the tubes or wells of interest and then centrifugation at 13,266g for 3 minutes. The supernatant was removed, and the pellet was re-suspended in 50 µL sterile distilled water, mixed using a vortex mixer and re-centrifuged at 13,266g for 3 minutes.

This step was repeated once more to ensure complete removal of growth media from the cells, and to reduce antifungal carry-over to the subsequent extraction and analysis steps (Saracli *et al*., 2015).

2.5.3 MALDI-TOF MS analysis

Prepared, matrix covered protein extracts on target plates (Figure 1) were placed into a Bruker MicroFlex LT bench top MALDI-TOF MS platform (Bruker Daltonic GmbH, Bremen, Germany) according to the manufacturer's instructions. Standard analysis was performed using the default laser frequency at 60Hz for every run, in linear positive ion acquisition mode. The default instrument settings for recommended clinical laboratory use included: lens, 8.5kV; ion source 1, 20kV; ion source 2, 18.1kV; with a detectable mass range of 2,000 – 20,000-Da. For each sample tested a minimum of 240 laser shots was used to create spectral data, with 40 laser shots at six randomised positions on a single target plate spot and the combined sum spectra was analysed in various software packages as detailed in Section 2.6.2.

2.5.4 Creation of main spectrum profiles (MSP) for inclusion in the MALDI-TOF MS database

In order to create a representative main spectrum profile (MSP) as previously described (Fraser *et al*., 2016). Briefly, eight replicates of the extracted proteins from cultures that were 24 hours old were measured in triplicate using Bruker's FlexControl software. This generates a profile consisting of 24 spectral records for the organism of interest (Figure 10). Using Bruker's FlexAnalysis software, the consistency and reproducibility

of all 24 replicates is inspected and evaluated (Figure 11). Once the spectra have been validated a third piece of software, Biotyper 3.1, is used to compare each spectrum to existing MSP spectra in the available MALDI-TOF MS databases. Once again, the data is interrogated for possible matches and overlap which may negate the creation of a new MSP record within the database (Figure 12). In the event that no overlap is manually observed, Biotyper 3.1 allows the creation of an MSP from the spectral dataset. This can then be catalogued and entered into the taxonomic tree of the MRLDB or other internally curated database.

2.6 Data Analysis

Multiple commercially available software packages were used to visualise and integrate the data generated throughout this study. In keeping with the full remit of this study, all the software programs are part of the standard installation package for the machine or platform provided by the manufacturer at purchase. An exception was the 4Peaks software which is a tool designed to read specific file formats generated by sequencing analysis machines. For ease of acknowledgment, all are listed here:

2.6.1 Genomic analysis

DNA sequence analysis: **4Peaks** designed by A. Griekspoor and Tom Groothuis, www.nucleobytes.com

DNA sequence matching: **BLASTn** online tool, www.blast.ncbi.nlm.nih.gov

Pyrosequencing operation: **PyroMark™ID** created by Biotage AB, Uppsala, Sweden

Pyrosequencing sequence analysis: **IdentiFire®** created by Biotage AB, Uppsala, Sweden

2.6.2 Proteomic analysis

LC-MS/MS

Operation: **Xcalibur 2.1** (Thermo Scientific, USA)

Protein identification: **ProteinPlot** and www.uniprot.org

SEQUEST (Thermo Scientific, USA)

MALDI-TOF MS

Compass for Flex Series 1.3 including;

Operation: **FlexControl 3.3** (Bruker Daltonic GmBH, Bremen, Germany)

Analysis: **Biotyper 3.1** and **FlexAnalysis** (Bruker Daltonic GmBH, Bremen, Germany)

Statistical analysis of spectral data was conducted within the Biotyper 3.1 software. The functions for the creation of principal component analysis (PCA) and composite correlation indices (CCI) are built into the Biotyper 3.1 software package as a tool for enhanced analysis. However, the software does not provide detail as to the elements of data which are utilised in their calculation, and their use in this study was primarily to demonstrate a different visual representation of the spectral data.

2.7 Ethical approval and risk assessment

This research was conducted at the Public Health England National Mycology Reference Laboratory (PHE MRL) in Bristol. Whilst there were already appropriate risk assessments in place for all the activities undertaken as part of this study, a new risk assessment for the complete undertakings within the study was completed and approved by onsite health and safety management.

Full ethical and research governance was approved for this project by the PHE Research and Development office (R&D243) and by the UWE ethics committee (UWE REC REF No: HAS.15.08.004). There were no human participants within this study and only archived clinical isolates of microorganisms were used, with no linkage to patient medical records. There was therefore no requirement for patient information, and as such no personal data was stored or collected. Letters of confirmation of approval to proceed can be found in Appendix I.

3. RESULTS

3.1 Detection of genetic markers of resistance

3.1.1 Collection/selection of isolates and susceptibility testing

Initial isolates of *C. glabrata* were selected for this study based upon their accession into the NCPF as CSP resistant isolates from clinical samples. The initial selected isolates were NCPF 8714, 8715, 8745, 8814 and 8919. Once fully revived, all isolates were subjected to confirmatory echinocandin resistance testing by E-test as described in Section 2.1 and the results are shown in Table 6. It is clearly highlighted in italics in Table 6 that only 2/5 isolates revived from the NCPF were deemed resistant according to the breakpoints used throughout the timeframe of this study (Fraser *et al*., 2019a; CLSI, 2017a; CLSI 2017b). Discussion and interpretation of this finding can be found in Section 4.2. NCPF 8714 and 8715 were discontinued from further study and NCPF 8745 was used throughout the study as an echinocandin susceptible isolate.

Table 6. Minimum inhibitory concentration (MIC, µg/mL) of caspofungin as demonstrated by E-test for revived NCPF isolates Table presenting the MIC values as performed by E-test for caspofungin of isolates of *C. glabrata* revived from the NCPF. All isolates entered the collection as echinocandin resistant. Section 4.2 provides discussion of discrepancies in the expected resistance highlighted here (*Italics*).

Key: MIC = minimum inhibitory concentration, NCPF = National Collection of Pathogenic Fungi.

NCPF Isolate	**8714**	**8715**	**8745**	**8814**	**8919**
MIC Result µg/mL (24 hours)	*0.064*	*0.064*	*0.016*	6	>32
MIC Result µg/mL (48 hours)	*0.064*	*0.064*	*0.016*	>32	>32

During the period of this study, between July 2015 and July 2018, a total of 2713 clinical isolates of *C. glabrata* were submitted to the PHE MRL for antifungal susceptibility testing. Isolates that demonstrated some form of elevated echinocandin MIC or were classified as exhibiting intermediate susceptiblity or resistance to one or more echinocandin agent as determined by CLSI microbroth dilution (Table 2; CLSI, 2017a; 2017b) were admitted into this study. This totalled fifteen (0.55%) clinical isolates and these were assigned a study (clinical) number accordingly, the results of the susceptibility testing are found in Table 7.

Table 7 <u>Minimum inhibitory concentration (MIC, µg/mL) of echinocandin for all isolates used during this study between July 2015 – July 2018.</u> Table provides the data for the archived echinocandin resistant isolates from the National Collection of Pathogenic Fungi (NCPF) and clinical isolates which exhibited echinocandin MICs classified as resistant or intermediate acquired during the period of study, July 2015 – July 2018 (Fraser et al., 2019

Key: MIC = minimum inhibitory concentration (µg/mL), NCPF = National Collection of Pathogenic Fungi, ANF = anidulafungin, CSP = caspofungin, MCF = micafungin, '-' = not tested.

	MIC (µg/mL)		
Isolate	**ANF**	**CSP**	**MCF**
<u>NCPF Number</u>			
8714	-	0.06	-
8715	-	0.06	-
8745	-	<0.015	-
8814	4	6	1
8919	6	>32	6
<u>Clinical Number</u>			
61	-	8	1
67	0.25	0.5	0.016
68	-	0.25	-
70	-	0.25	-
71	0.5	1	-
72	2	32	4
73	2	4	-
74	2	2	-
75	0.5	2	0.25
76	0.5	4	0.5
77	0.25	0.5	0.03
78	2	2	0.75
79	2	2	0.5
80	2	>16	2
81	0.5	2	0.125

3.1.2 Primer design and suitability for analysis of *FKS* regions

Traditional Sanger sequencing of the *FKS* region was applied to NCPF 8714 to determine the amplification of the correct region required for detection in this study with the primer set described in Table 3. The primary objective was to ensure that primers for each region (*FKS1* and *FKS2*) did not overlap and cross amplify. The resulting sequences were subjected to a publicly synchronised online nucleotide database search using BLASTn. The resulting matches correctly identified the sequence homology between the PCR product and the *FKS1* region in *C. glabrata*. This process was repeated for the *FKS2* region, with similar results. Example sequence traces, the respective BLASTn matches and interpretation can be found in Figures 2 - 5. This process was only performed on a single isolate of NCPF 8714 due to the financial constraints of outsourcing Sanger sequencing, which is no longer performed in the diagnostic laboratory.

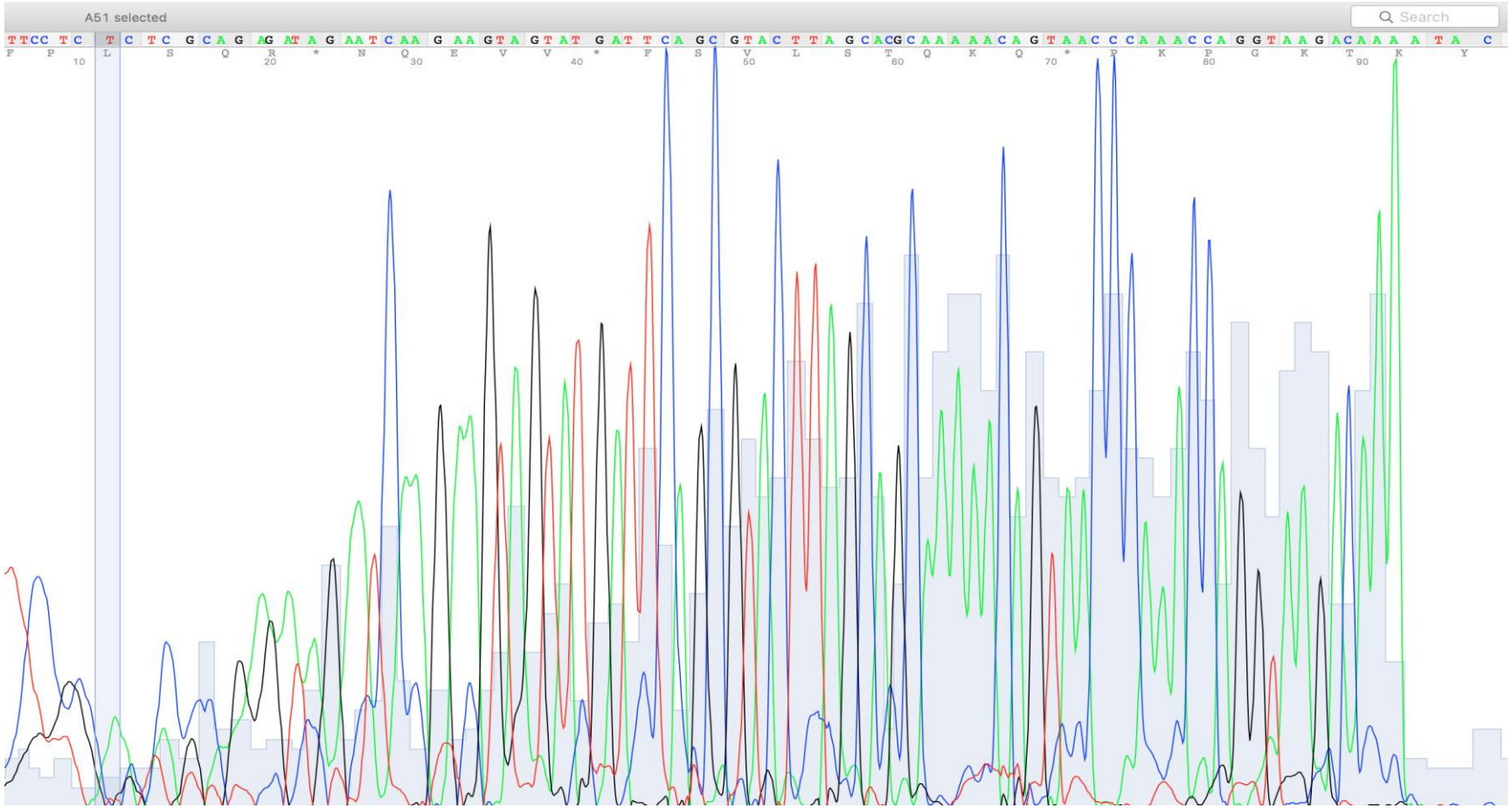

Figure 2. <u>Sanger sequencing trace for *C. glabrata* NCPF 8714 *FKS*1.</u> *C. glabrata* NCPF 8714 *FKS*1 (92 bases) visualised using 4Peaks sequencing software. Different colour peaks represent the addition of a new nucleotide into the sequencing strand. These are listed in the matching colour along the top. Key: Green = adenine (A), blue = cytosine (C), black = guanine (G), red = thymine (T).

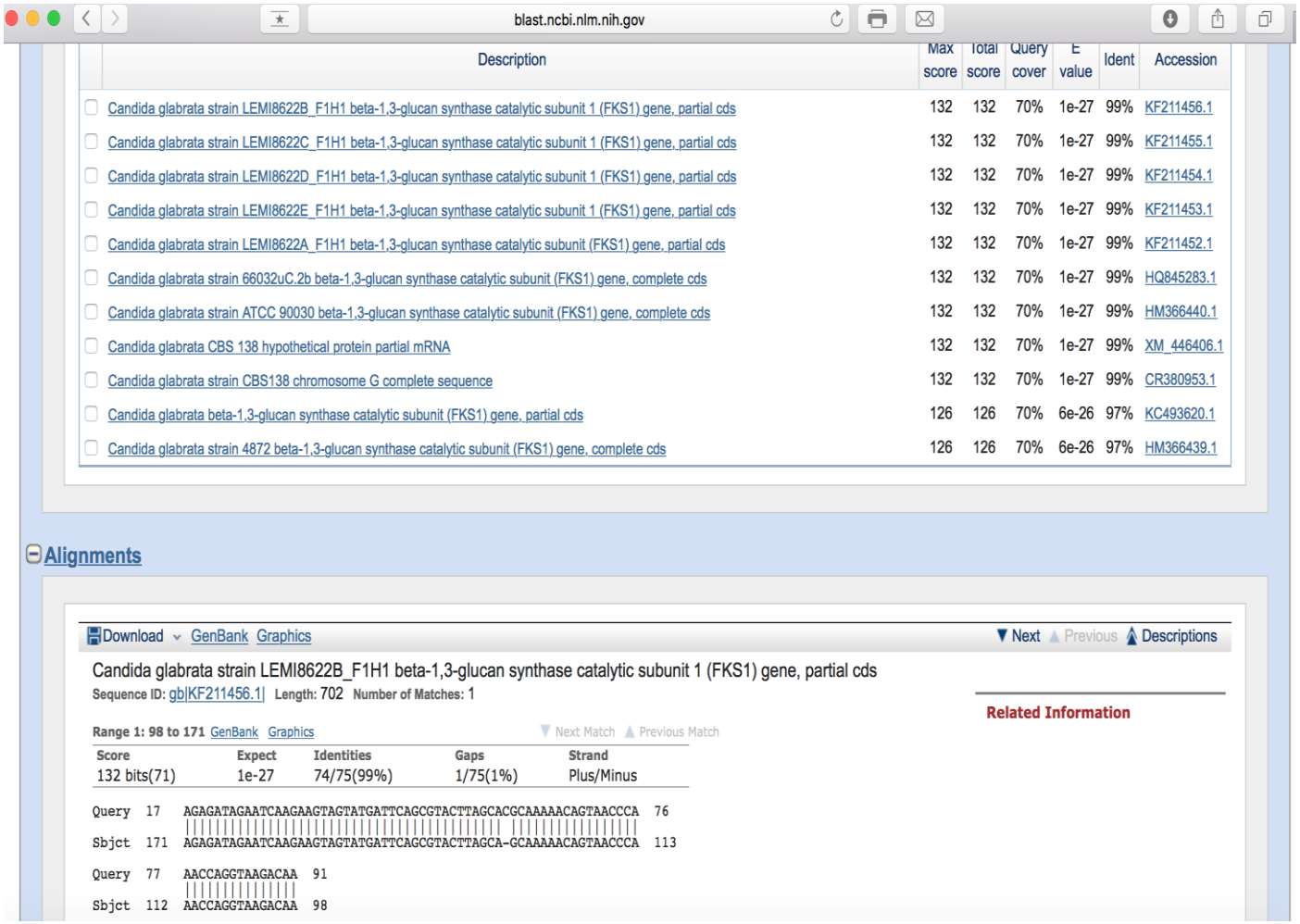

Figure 3. <u>BLASTn matches for *FKS1* primer.</u> BLASTn web-based search engine matches for *FKS1* primer with *C. glabrata*. Key: A = adenine, C = cytosine, G = guanine, T = thymine.

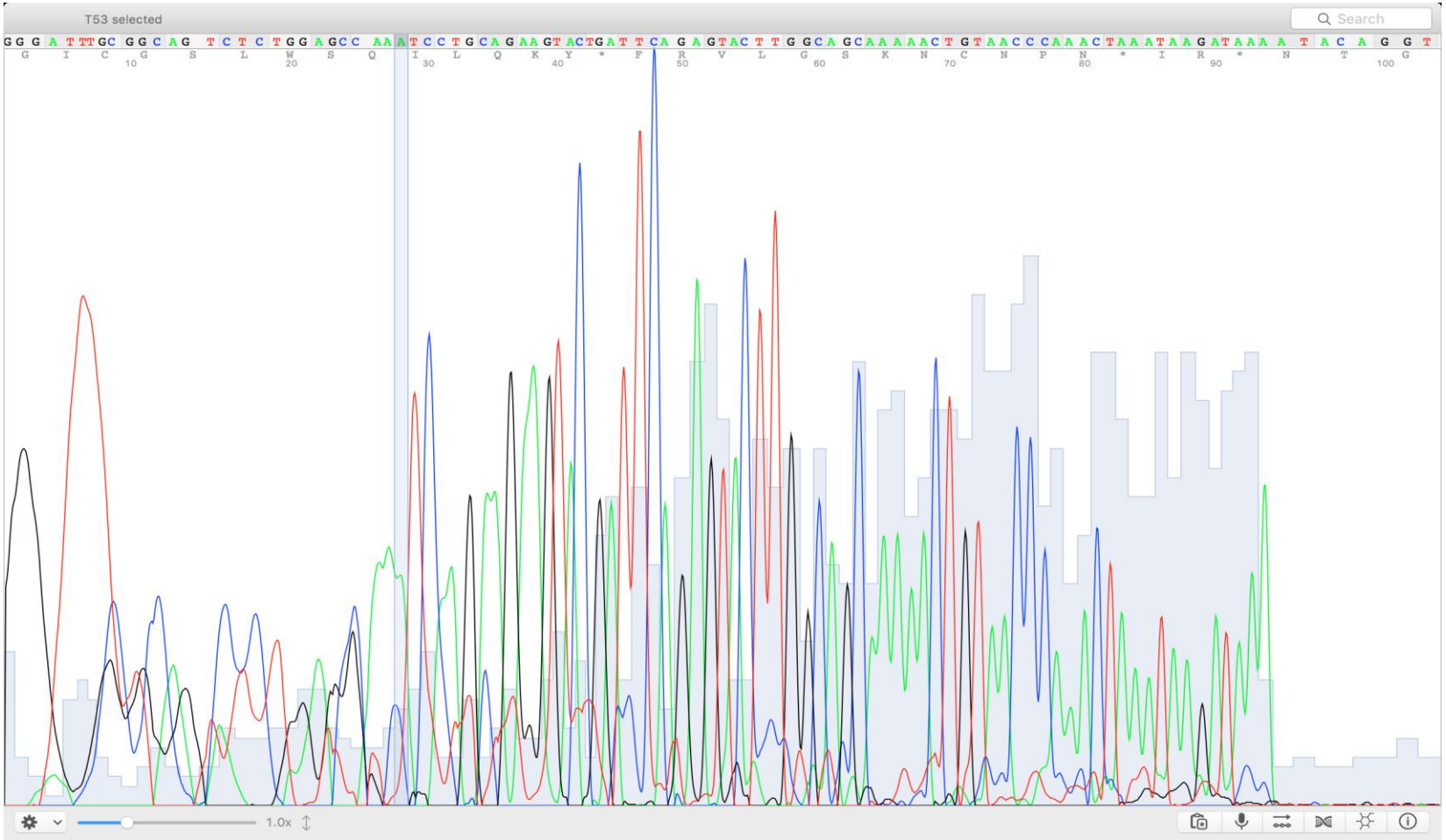

Figure 4. <u>Sanger sequencing trace for *C. glabrata* NCPF 8714 *FKS2*.</u> *C. glabrata* NCPF 8714 *FKS2* (109 bases) visualised using 4Peaks sequencing software. Different colour peaks represent the addition of a new nucleotide into the sequencing strand. These are listed in the matching colour along the top. Green = adenine (A), blue = cytosine (C), black = guanine (G), red = thymine (T).

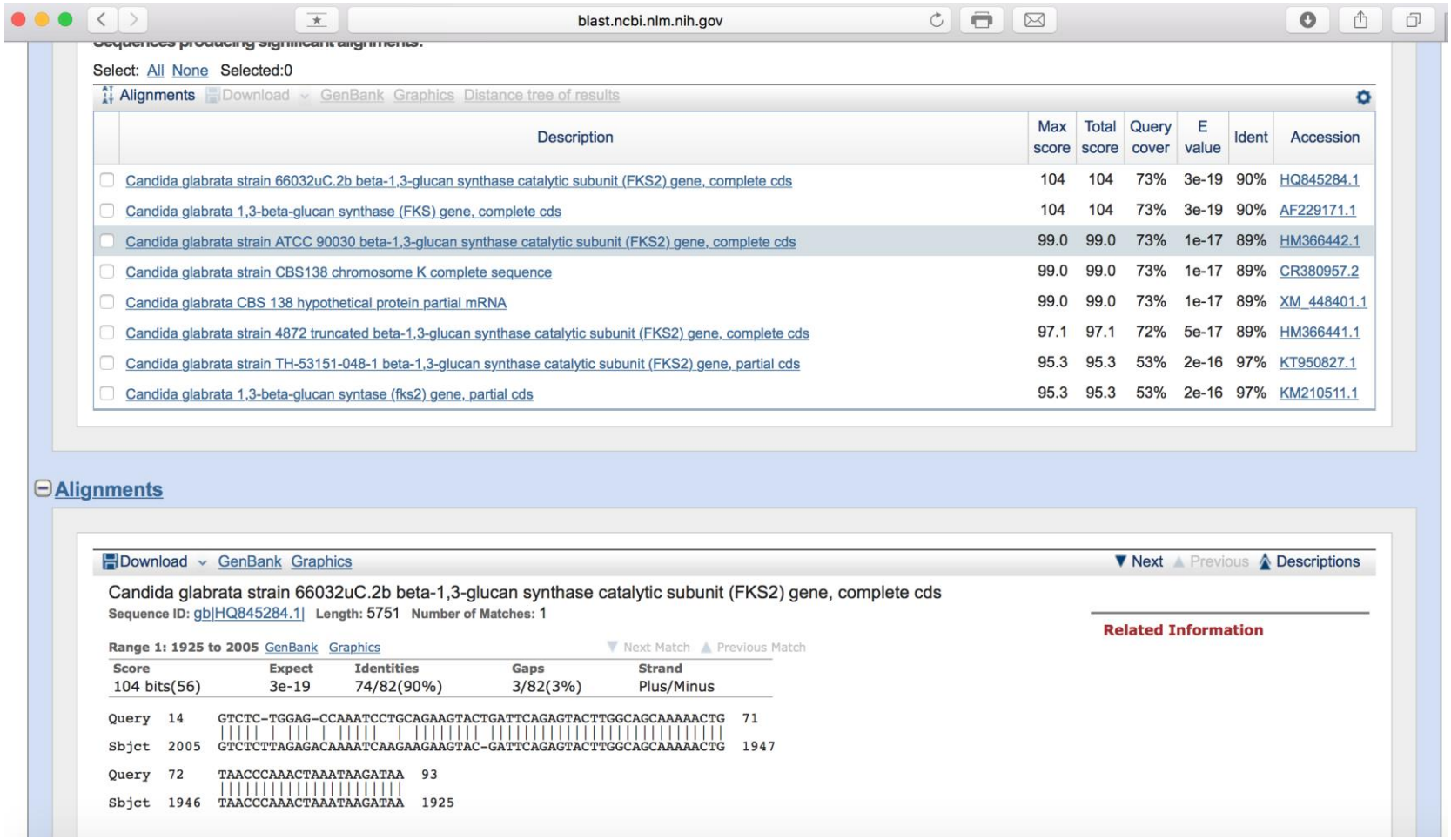

Figure 5. <u>BLASTn matches for *FKS2* primer.</u> BLASTn web-based search engine matches for *FKS2* primer with *C. glabrata*. Key: A = adenine, C = cytosine, G = guanine, T = thymine.

3.1.3 Pyrosequencing of *FKS* regions

Pyrosequencing of the NCPF reference isolates of *C. glabrata* resulted in the detection of two isolates (NCPF 8814 and NCPF 8919) with hot spot mutations and this correlated with phenotypic resistance to echinocandin agents as shown in Table 6 and Table 8. Figure 6 shows an example sequence profile generated by the IdentiFire® software and Table 8 details the results of the initial pyrosequencing of all the revived NCPF isolates. As shown in Table 8, wild type (no hot spot mutation) *FKS* sequences were detected for all NCPF isolates in *FKS1*, and for NCPF 8714, NCPF 8715 and NCPF 8745 in *FKS2*. The detection of a hot spot mutation for phenotypic resistant isolates NCPF 8814 and NCPF 8919 appears in *FKS2* and represents the substitution of a serine for a proline at position 663 (S663P). The full FASTA format sequences possible for both *FKS* regions used to create the database are available to view in Appendix II.

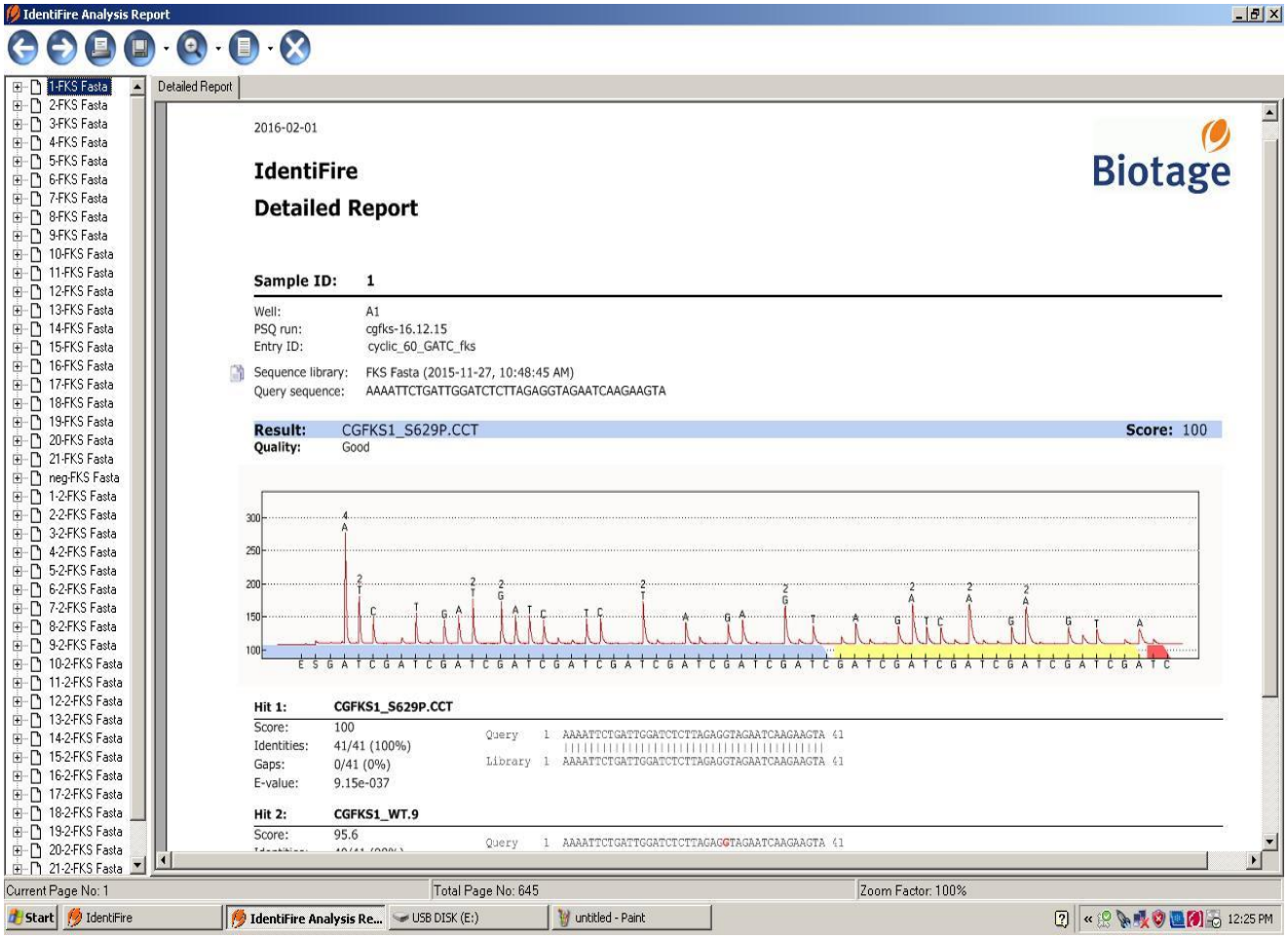

Figure 6. **<u>Pyrosequencing analysis report for *FKS* mutant.</u>** Example of sequence analysis report and *FKS* mutation identification using the IdentiFire® software and the *FKS* Fasta file (Appendix II). The red line identifies a successful nucleotide insertion into the query sequence, the relative size of the peak indicates multiple nucleotide insertions. Key: A= adenine, C = cytosine, G = guanine, T = thymine.

Table 8. <u>Pyrosequencing results for NCPF isolates of *C. glabrata*</u> Table demonstrates the pyrosequencing results of *FKS1* and *FKS2* for the NCPF isolates revived at the start of this study (Fraser *et al*., 2019a).

Key: Isolate = NCPF number; MIC = Echinocandin minimum inhibitory concentration at 24 hours (µg/mL); Class (Classification) = Susceptible (S) or Resistant (R); *FKS1*/*FKS2* = sequence match generated by IdentiFire® software; Mutation = No (N), or Yes (Y) and including the *FKS* gene (1/2), amino acid (Aa) substitution and gene position (XXX) in the format: 1/2 – AaXXXAa.

Isolate	MIC (µg/mL)	Class	*FKS1*	*FKS2*	Mutation
8714	0.064	S	CGFKS1_WT.3	CGFKS2_WT3	N
8715	0.064	S	CGFKS1_WT.3	CGFKS2_WT3	N
8745	0.016	S	CGFKS1_WT.3	CGFKS2_WT3	N
8814	6	R	CGFKS1_WT.3	CFKS2_S663P.CCT	Y, 2-S663P
8919	>32	R	CGFKS1_WT.2	CFKS2_S663P.CCT	Y, 2-S663P

3.1.4 *In situ* induction of resistance to echinocandin antifungal agents

Unexpectedly, three of the five revived NCPF isolates (NCPF 8714, NCPF 8715 and NCPF 8745) did not demonstrate any evidence of echinocandin resistance (Table 6 and Table 8), and this presented an opportunity to attempt the induction of resistance in these isolates using a process of serial passage and antifungal exposure. As described in section 2.2.5, the method used has been suggested as an accurate reflection of *in vivo* exposure to antifungal agents in a clinical setting (Zimbeck *et al*., 2010). Each serial sub-culture resulted in a unique isolate which was accessioned to a short-term storage system (SABC agar slope) so that if resistance was successfully induced, sequential alterations in the genetic or proteomic profiles could be detected and tracked through each individual passage. This would also enable the capture of those isolates that

demonstrate MIC values considered borderline, or indicating emergent resistance, therefore providing a more complete picture of the induction of resistance in the presence of antifungal agent.

Freshly grown isolates of NCPF 8714, NCPF 8715 and NCPF 8745 were subjected to serial sub-culture in the presence of ANF in an attempt to induce resistance as previously demonstrated with MCF (Bordallo-Cardona *et al*., 2017). The presence of macro-colonies within areas of inhibition was taken as indicative of resistant sub-populations and these macro-colonies were stored, re-tested and further sub-cultured. All macro-colonies were subjected to pyrosequencing analysis to determine *FKS* sequences. Table 9 presents a subset of the induction assay results demonstrating how each isolate was repeatedly sub-cultured and visible macro-colonies were removed and re-tested. The full data set is available in Appendix IV (Table 1). Isolate NCPF 8715 was the only isolate to demonstrate the appearance of potentially inducible resistance, whereby continual serial sub-cultures of this isolate resulted in the production of multiple macro-colonies with varying MIC values. However, no detectable *FKS* mutations were found by pyrosequencing in any of the sub-cultured isolates collected at any stage of passage. This may suggest that the subcultures which exhibited the appearance of microcolonies within zones of inhibition did not actually represent induced resistance in this instance. In fact, on further examination this culture had become contaminated at some point with an isolate of *C. parapsilosis* for which the MIC cut off value for susceptibility is higher (≤2.0 μg/mL; CLSI, 2017b*)*. The full data set was therefore omitted from further study.

Table 9. <u>Sub-culture and resulting macro-colonies of NCPF isolates exhibiting decreased susceptibility to anidulafungin</u>. Table displays the pyrosequencing results for the NCPF isolates that demonstrated the presence of macro-colonies potentially indicative of induced resistance on serial sub-culture. The full data set is available in Appendix IV (Table 1).

Key: Isolate = NCPF isolates, or macro colony identifier; Sub = number of sub-culture; Macro = number of macro-colony on sub-culture; MIC = minimum inhibitory concentration of anidulafungin at 24 hours, or in case of multiple isolates a range is provided (µg/mL); *FKS*1/2 = Identifire® sequence identification; Mutation = No (N), or Yes (Y) and including the *FKS* gene (1/2). Full table can be found in Appendix IV

Isolate	Sub	Macro	MIC (µg/mL)	*FKS1*	*FKS2*	Mutation
8714	6	0	0.012	CGFKS1_WT.3	CGFKS2_WT3	N
8715	2	1	0.5	CGFKS1_WT.3	CGFKS2_WT3	N
8715 sub2-macro1	2	7	0.25 - 4	CGFKS2_WT3	CGFKS2_WT3	N
8715 sub2-macro4	1	2	1-2	CGFKS2_WT3	CGFKS2_WT3	N
8715 sub2-macro6	1	4	1-2	CGFKS2_WT3	CGFKS2_WT3	N
8745	6	0	0.016	CGFKS1_WT.3	CGFKS2_WT3	N

3.1.5 Application to clinical isolates demonstrating phenotypic resistance

Table 10 shows the pyrosequencing results obtained for clinical isolates submitted for routine susceptibility testing to PHE MRL during the timescale of this study that had demonstrated phenotypic resistance to one or more echinocandin agents by *in vitro* microbroth dilution testing. A hot spot mutation representative of a substitution of serine to proline at position 629 was detected in *FKS1* for clinical isolates 61, 71, 72, 75, 76 and 81. For *FKS2*, conclusive hot spot mutations representing a substitution of serine for proline at position 663 were detected in clinical isolates 73, 74, 78 and 79. Isolates 71 and 72 may have mutations in both *FKS1* and *FKS2* but despite repeat testing, the sequence homology score for *FKS2* was never greater than 90%. Isolates 61 and 80 failed to show any evidence of amplification for the *FKS2* region even after repeated attempts and are listed as inconclusive for mutations in this region. In the case of isolate 80, *FKS1* demonstrated a wild type sequence, suggesting the potential that the resistance mechanism for this isolate may not reside in *FKS* at all. Without the ability to verify the *FKS2* region, this cannot be proven at this stage.

Table 10. *FKS* results for clinical isolates showing *in vitro* resistance. Table shows clinical submissions to PHE MRL that demonstrated phenotypic resistance to one or more echinocandin agent during the study period and the pyrosequencing result returned. This is provided as the mutation or wild type (WT) sequence, and if the sequence homology was >100%, the percentage match is provided (Fraser *et al*., 2019a).

Key: MIC = Minimum inhibitory concentration (µg/mL), ANF = anidulafungin, CSP = caspofungin, MCF = micafungin, na = not available, WT# = Wild Type sequence number, X###X = amino acid, position, amino acid substitute, "-" = Inconclusive, *sequence homology score <100.

Clinical Isolate Number	MIC (µg/mL) ANF/CSP/MCF	*FKS1*	*FKS2*
61	na/8/1	S629P	-
67	0.25/0.5/0.016	WT3	WT2
68	na/0.25/na	WT3	WT2
70	na/0.25/na	WT3	WT2
71	0.5/1/na	S629P	?S663F 83.1%*
72	2/32/4	S629P	?S663F 77.4%*
73	2/4/na	WT3	S663P
74	2/2/na	WT3	S663P
75	0.5/2/0.25	S629P	WT3
76	0.5/4/0.5	S629P	WT3
77	0.25/0.5/0.03	WT3	WT3
78	2/2/0.75	WT3	S663P
79	2/2/0.5	WT3	S663P
80	2/>16/2	WT3	-
81	0.5/2/0.125	S629P	WT3

3.2 Detection of proteins by LC-MS/MS that may indicate resistance

3.2.1 LC-MS/MS analysis of protein extracts

LC-MS/MS was performed on prepared extracts of *C. glabrata* isolates NCPF 8814 (echinocandin resistant, *FKS* mutation present) and NCPF 8745 (echinocandin susceptible), at the proteomics facility of the University of Bristol, to compare and characterise proteins present within both isolates. Two different protein extracts were compared: one extracted using a protocol optimised for LC-MS/MS techniques (Extract A) and the other using an extraction protocol specifically created for MALDI-TOF MS identification (Extract B) as described in Section 2.4. Once extracted, the prepared suspensions requiring LC- MS/MS analysis were forwarded to the proteomics facility, University of Bristol. This series of experiments was only performed once, and on a single example of each isolate. This was due to the high complexity of analysis and cost of outsourcing.

Tables 12 and 13 represent some examples of the data analysis provided by the LC-MS/MS analysis. The primary aim of using LC-MS/MS was to provide an indication of the potential protein targets related to echinocandin resistance within an extracted preparation of *C. glabrata*. This may have taken the form of a loss of or reduction in abundance of an existing protein, or the creation of a new protein. It was therefore essential to compare an echinocandin resistant isolate alongside an echinocandin susceptible isolate. Extraction protocols were developed for LC-MS/MS and MALDI-TOF MS so that they could be compared as to the relative quantity of information provided by each extraction method. Table 11 provides an overview of the total number of predicted proteins present in both extractions for each isolate. These data are sorted

to identify proteins which may be unique to either the echinocandin resistant isolate (NCPF 8814) or the echinocandin susceptible isolate (NCPF 8745).

For NCPF 8745, a total of 1379 proteins were predicted by peptide fragment analysis in extract A, and 296 in extract B. Of these proteins, 1160 (84.1%) were only found in extract A and 2 (0.17%) of those were unique to NCPF 8745. In extract B, 77 (26.0%) proteins were unique to the extraction method with 16 (7.8%) of these being identified as appearing only in NCPF 8745.

For NCPF 8814, a total of 1486 proteins were suggested in extract A and 248 in extract B. Of these predicted proteins, 1278 (86%) were identified as being unique to extract A with a total of 7 (0.55%) being specific to NCPF 8814 and 40 (16.1%) were found to present in extract B alone, with 5 (12.5%) representing unique proteins to NCPF 8814. Table 13 shows all the unique proteins predicted in the MALDI-TOF MS extraction (extract B) with NCPF 8814, the echinocandin resistant isolate, which were not detected in the same extraction for NCPF 8745, the echinocandin susceptible isolate. Four of the predicted proteins represent ribosomal proteins which could be detectable by MALDI-TOF MS (Clark *et al*., 2013). This serves to highlight their potential utility as markers of resistance when using MALDI-TOF MS in the clinical diagnostic laboratory. Table 13 provides a complete list of all the predicted proteins present in the MALDI-TOF MS extract for NCPF 8814. Whilst these predicted proteins are not necessarily specific to the echinocandin resistant isolate alone, they provide a much larger selection of probable markers or indicators of resistance for analysis by MALDI-TOF MS. However, a direct consequence of the shot gun approach to proteomics adopted over the past decade results in 30 (75%) of the potential predicted targets being returned as uncharacterised proteins with differing roles in many aspects of cell biology as inferred from the homology of predicted proteins in Tables 12 and 13. The complete LC-MS/MS

data sets for each extraction technique, with each isolate are accessible via the UWE data repository (Fraser, 2019b, available from: http://researchdata.uwe.ac.uk/444).

Table 11. <u>The number of whole proteins predicted from peptide sequence spectra generated by LC-MS/MS.</u> Isolates NCPF 8745 (echinocandin susceptible) and NCPF 8814 (echinocandin resistant) are presented with an overview of the number of proteins predicted from peptide sequence spectra generated by LC-MS/MS allowing the comparison of relative protein recovery for each extraction. Extraction A is specifically modified for LC-MS/MS analysis, and Extraction B is the standard extraction used for routine yeast identification by MALDI-TOF MS.

	Total number of predicted proteins	**Mass Range of Proteins (Da)**	**Total number of proteins unique to extraction type**	**Total number of predicted proteins identified as unique to isolate**
<u>Extract A</u>				
8745	**1379**	**8000 – 432,200**	**1160**	**2**
8814	***1486***	***6400 – 352,600***	***1278***	***7***
<u>Extract B</u>				
8745	**296**	**6400 – 118,400**	**77**	**16**
8814	***248***	***6300 – 68,800***	***40***	***5***

Table 12. <u>Predicted unique proteins from echinocandin resistant isolate, NCPF 8814</u>. Table showing predicted unique proteins not recovered in extracts of echinocandin susceptible isolate, NCPF 8745 but detected by both extraction methods at 1% false discovery rate.

Key: MW = molecular weight, H = inferred from homology in similar organisms, P = predicted by previous proteomic studies.

Protein	MW (Da)	Protein Discoverer™	UniProt	Molecular function (if known)
F2Z629	9900	Ribosomal Protein L37	Ribosomal Protein L37	Metal ion binding, rRNA binding, structural component of ribosome -2 (H)
Q6FQH1	10900	Uncharacterised	Cytochrome b-C1	Ubiquinol-cytochrome-c reductase activity -1 (P)
Q6FXR7	13600	Uncharacterised	37S ribosomal protein YMR-31, mitochondrial	Ribonucleoprotein, ribosomal protein – 1 (P)
Q6FW92	15500	54S ribosomal protein L31	54S ribosomal protein L31, mitochondrial	Structural component of ribosome -1 (P)
Q6FRV9	22100	Uncharacterised	40S ribosomal protein S9-A	rRNA binding, structural component of ribosome – 1 (P)

Table 13. <u>Proteins predicted by LC-MS/MS from NCPF 8814 which are only present in MALDI-TOF MS extraction.</u> Proteins as predicted by LC-MS/MS present only in extract B (MALDI-TOF MS specific) of echinocandin resistant isolate NCPF 8814, at 1% false discovery rate. This identifies their potential use as resistance markers in MALDI-TOF MS.

Key: MW= Molecular weight, H = inferred from homology in similar organisms, P = predicted by previous proteomic studies.

Protein	MW (Da)	Protein Discoverer™	UniProt	Molecular Function (if known)
B4UN51	6300	Uncharacterised	Uncharacterised	Structural constituent of ribosome (H)
B4UN07	7100	Uncharacterised	Uncharacterised	Cytochrome-c oxidase activity (P)
Q6FVK6	7600	Uncharacterised	Uncharacterised	(P)
Q6FT27	7800	Uncharacterised	Uncharacterised	DNA binding, RNA polymerase activity, zinc ion binding (P)
B4UMX6	7900	Uncharacterised	Uncharacterised	(P)
B4UN08	8000	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
B4UMY3	8300	Uncharacterised	Uncharacterised	Cytochrome-c oxidase activity (P)
B4UMX5	8500	Uncharacterised	Uncharacterised	Transmembrane (P)
B4UMX9	9400	Uncharacterised	Uncharacterised	(P)
Q6FVQ3	9700	Cytochrome c oxidase assembly protein COX19	Cytochrome c oxidase assembly protein COX19	Copper ion binding (H)
B4UN34	10700	Stationary phase protein 4	Stationary phase protein 4 SPG4	Stationary phase essential protein (H)
Q6FP60	10800	Uncharacterised	Uncharacterised	ATPase inhibitor activity (P)
Q6FMK4	10800	Uncharacterised	Uncharacterised	Cytochrome-c oxidase activity (P)
B4UN26	11000	Uncharacterised	Uncharacterised	Unknown
B4UN65	11300	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FQ40	11700	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FLM0	13000	Uncharacterised	Uncharacterised	Tubulin binding, unfolded protein binding (P)
Q6FQX5	13400	Cytochrome c oxidase-assembly factor COX23, mitochondrial	Cytochrome c oxidase-assembly factor COX23, mitochondrial	Mitochondrial respiratory chain complex assembly (H)

Table 13 continued.

Q6FIV0	13500	Long chronological lifespan protein 2	Long chronological lifespan protein 2 LCL2	Probable component of the endoplasmic reticulum-associated degradation pathway (H)
Q6FKT9	13500	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FQ39	15200	Uncharacterised	Uncharacterised	Mitochondrial respiratory chain complex II assembly (P)
Q6FT17	15400	Uncharacterised	mRNA stability protein	Protein serine/threonine phosphatase inhibitor activity (H), prevents degradation of specific nutrient-regulated mRNAs
Q6FL06	15800	Uncharacterised	Uncharacterised	7S RNA binding, endoplasmic reticulum signal peptide binding (P)
Q6FQS0	16100	Uncharacterised	Uncharacterised	(P)
Q6FST7	16500	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FUH0	16700	Uncharacterised	Uncharacterised, DAP1	Enzyme activator activity, heme binding (H)
Q6FMP3	16900	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FMW3	17000	Uncharacterised	Uncharacterised	(P)
Q6FNZ3	17200	Uncharacterised	Uncharacterised	mRNA binding, single stranded DNA binding, translation regulator activity, zinc ion binding (P)
Q6FJP2	17400	Uncharacterised	Uncharacterised	Thiosulphate sulphurtransferase activity (P)
Q6FVB3	17600	Uncharacterised	Uncharacterised	(P)
Q6FT71	20300	Actin-related protein 2/3 complex subunit 3	Actin-related protein 2/3 complex subunit 3	Actin binding, mediates the formation of branched actin networks (H)
Q6FIK8	20700	Uncharacterised	Uncharacterised	(P)
Q6FUK7	23700	Uncharacterised	Uncharacterised	Structural component of ribosome (P)
Q6FTI3	24100	Uncharacterised	Uncharacterised	(P)

Table 13 continued.

Q6FQ63	24100	Uncharacterised	Clathrin light chain	Structural molecule activity, clathrin is the major protein of the polyhedral coat of coated pits and vesicles (H)
Q6FS90	28000	Uncharacterised	Uncharacterised	(P)
Q6FIT0	30300	Uncharacterised	Cytochrome-c heme lyase	Holocytochrome –c synthase activity, covalently links the heme group to the apoprotein of cytochrome-c (H)
Q6FP52	30300	Signal recognition particle SEC65 subunit	Signal recognition particle SEC65 subunit	7S RNA binding, signal recognition particle, crucial role in targeting secretory proteins to the rough endoplasmic reticulum membrane
Q6FR69	68800	Uncharacterised Protein	Uncharacterised	Glutathione hydrolase activity (P)

3.2.2 Detection of predicted proteins by LC- MS/MS using MALDI-TOF MS

The data compiled from LC-MS/MS analysis as presented in Tables 11 - 13 demonstrated the presence of several possible protein indicators for resistance between the susceptible and resistant isolates. None of those predicted and characterised by the available software appears to have an existing recognised role in echinocandin resistance, whereby the majority of predicted proteins appear to be related to ribosomal structure and metabolic pathways. However, identification of organisms by MALDI-TOF MS is generally dependent upon ribosomal proteins present within the cytoplasm (Clark *et al*., 2013). The next step was to determine if any of these predicted potential protein targets could be visualised by MALDI-TOF MS under conditions used in the clinical laboratory to identify yeast isolates. In MALDI-TOF MS analysis, it is generally accepted that charge is equal to +1 therefore the atomic mass unit denotes molecular weight with accuracy generally accepted as 0.01% (Liu and Schey, 2005).
Figure 7 shows an example of MALDI-TOF MS spectra generated for both resistant (NCPF 8814) and susceptible (NCPF 8745) *C. glabrata* isolates with extract B, the standard MALDI-TOF MS extraction protocol for yeast identification in the clinical laboratory at the PHE MRL (Fraser *et al*., 2016). It can be seen that a large amount of spectral data appears within a relatively narrow mass detection window, between 2000 and 6000 Da. There appears to be a relative increase in the number of identifiable peaks presented by NCPF 8814 within this window when compared to NCPF 8745 as demonstrated by the presence of more peaks.

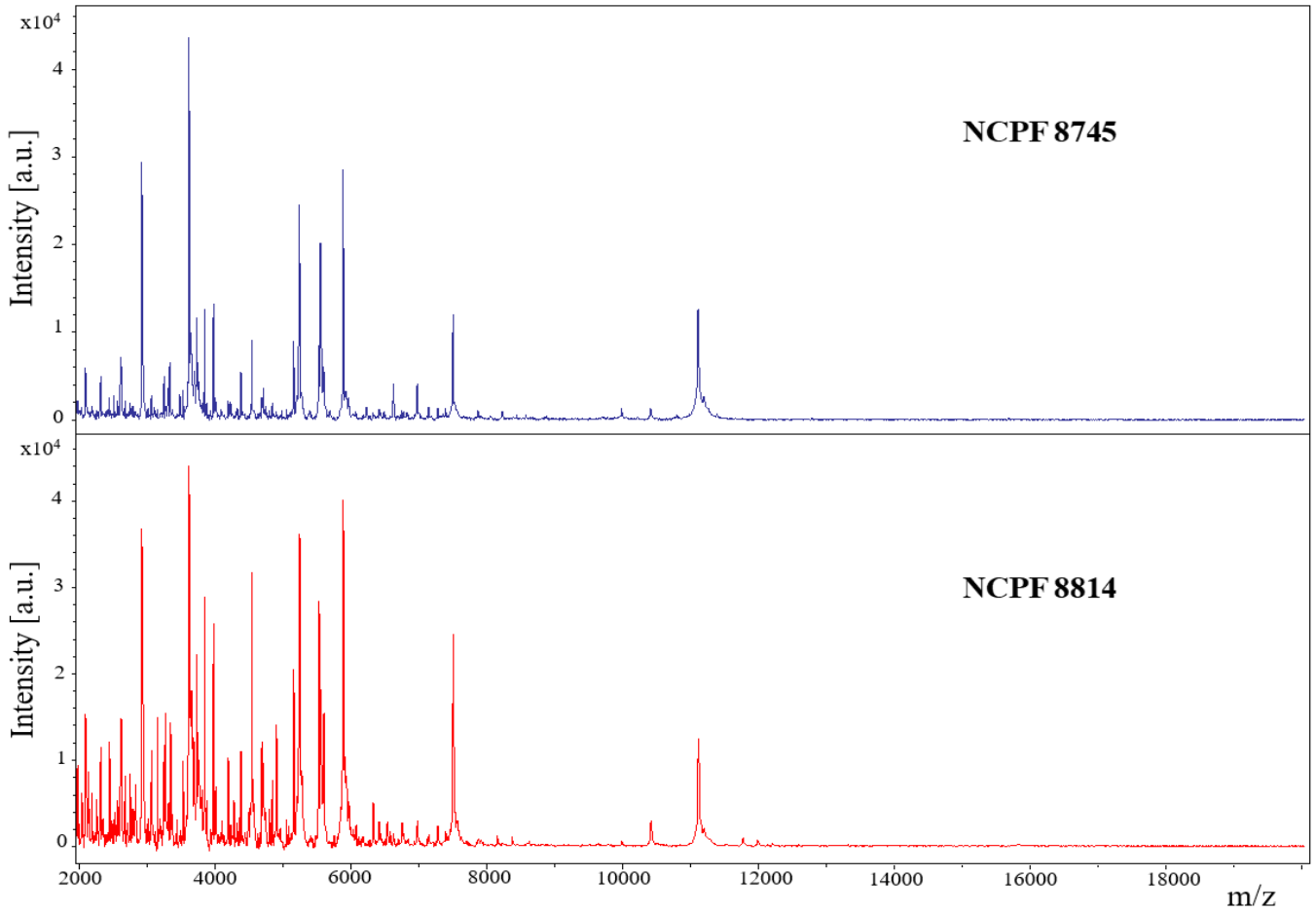

Figure 7. <u>MALDI-TOF MS protein spectra for NCPF 8745 and NCPF 8814 *C. glabrata* isolates</u>. This image shows MALDI-TOF MS spectra for *C. glabrata*; NCPF 8745 (blue spectrum) echinocandin susceptible, NCPF 8814 (red spectrum) echinocandin resistant over a mass range of 0-20,000 Da, as m/z after 24 hours growth on SABC agar. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary, m/z = mass/charge ratio. (Author's own image)

3.2.3 The effect of widening mass detection window in MALDI-TOF MS

As 7/40 (17.5%) of the proteins predicted by LC-MS/MS analysis present in extract B were outside of the 200-20,000 Da. mass range used for the identification of microbes by MALDI-TOF MS (Table 11), it suggested that the parameters for ionisation may require optimisation prior to accepting that no unique mass peaks were detectable on the MALDI-TOF MS platform. Therefore, the mass detection range was altered to encompass mass from between 0-70,000 Da. This window was limited by the available detection window of the Bruker MicroFlex LT, the MALDI-TOF MS machine currently in use at the PHE MRL. This value is within the operational maximum for the Bruker MicroFlex LT benchtop MALDI-TOF MS (0-100,000 Da). With reference to Tables 12 and 13 when looking for unique identification peaks using the MALDI-TOF MS extraction and the echinocandin resistant isolate NCPF 8814, the selected expanded mass range should be sufficient to indicate whether such an alteration in parameters could provide detection of the potential protein targets identified by LC-MS/MS.

Figure 8 shows the mass spectra acquired during an expanded mass detection window. It is clearly noticeable that the majority of identification peaks are compressed into a smaller section of the mass detection window, which makes it more difficult to distinguish individual mass targets. Figure 8 also demonstrates that no visible mass data is collected at values above 20,000 Da. Referring to Table 13, this indicates that the potential markers of resistance detectable in extract B is reduced from 40 to 33, as 7 of the predicted proteins have masses greater than 20,000 Da. Conversely, Table 12 indicates that of those predicted proteins that appear unique to NCPF 8814, the echinocandin resistant isolate, only 1 (Q6FRV9, 22100 Da, uncharacterised, possible rRNA binding protein) might not be captured by widening the mass detection window.

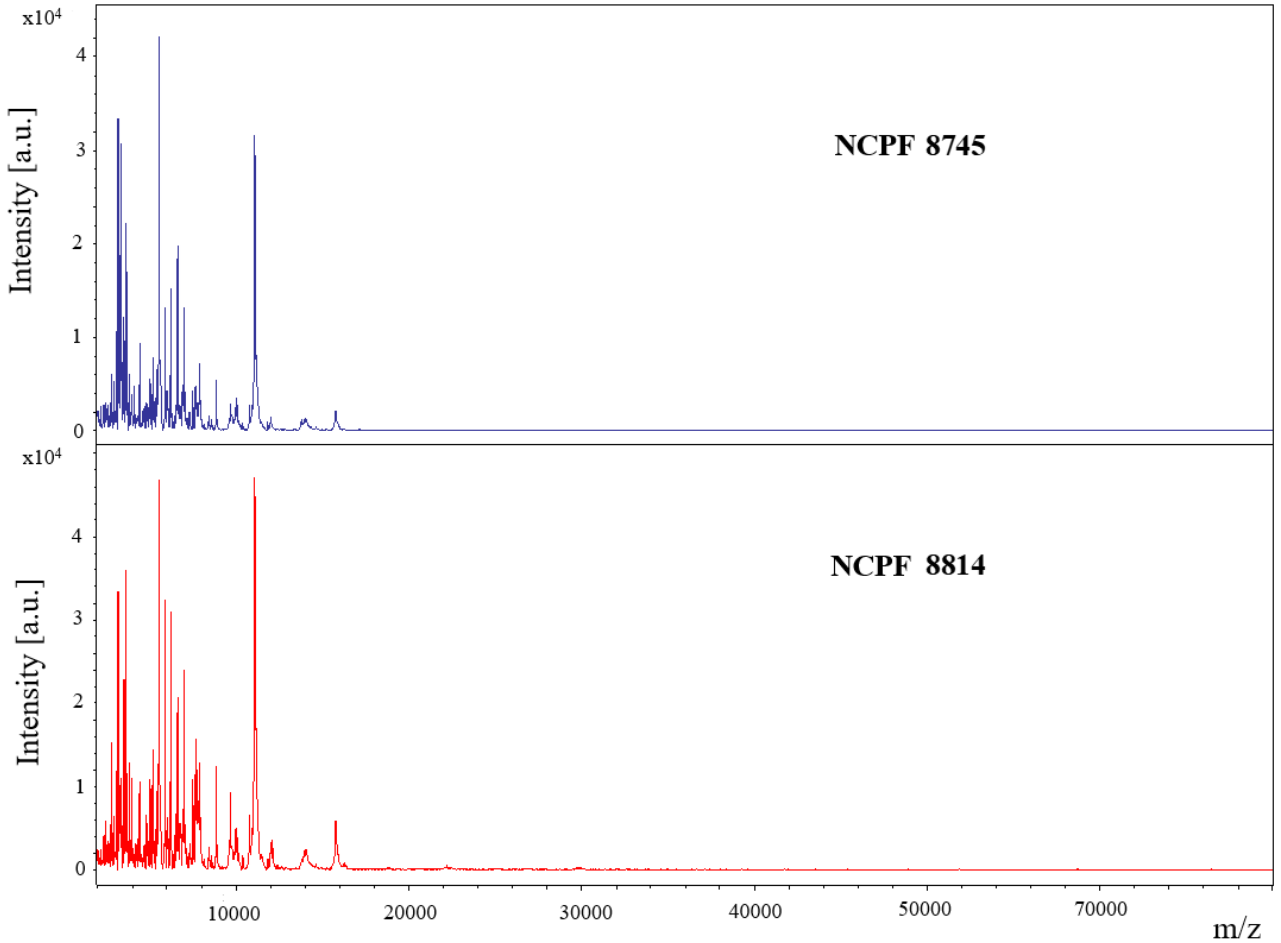

Figure 8. <u>Example of spectra generated by an increased mass detection window using FlexAnalysis software.</u> This image demonstrates the effect of an increased mass detection window (0 -70,000 Da, as m/z) for *C. glabrata*; NCPF 8745 (blue spectrum) echinocandin susceptible, NCPF 8814 (red spectrum) echinocandin resistant, as m/z after 24 hours growth on SABC agar. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] arbitrary unit, m/z = mass/charge ratio. (Author's own image)

3.2.4 The use of a different matrix for the ionisation of high molecular weight proteins

Extract A (LC-MS/MS specific), when analysed under the same conditions for both isolates, resulted in flat line (zero sum) spectra (Figure 9) despite repeated attempts. This suggested that the peptide fragments generated by the LC-MS/MS extraction were not detectable under the standard parameters used in the clinical laboratory for MALDI-TOF MS microbial identification.

As presented in Table 11, for NCPF 8814, a total of 248 predicted proteins were detected in extraction B by LC-MS/MS, and of those 40 did not appear in extraction A, indicating a unique presence in the MALDI-TOF MS extraction. However, none of these predicted proteins appeared to directly match any of the mass peaks present within the MALDI-TOF MS spectra, even allowing a 0.01% error for mass accuracy as suggested by Liu and Schey (2005).

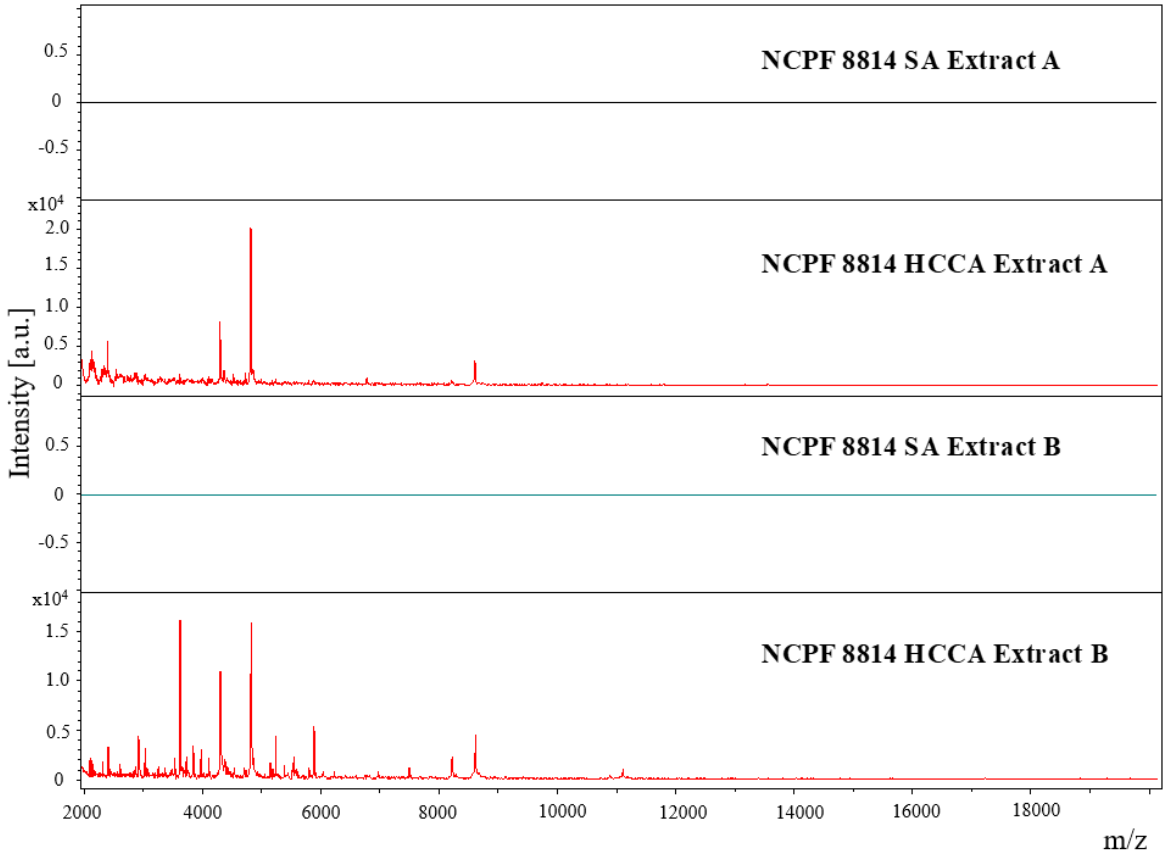

Figure 9. **Example spectra generated when using sinapinic acid as the matrix.** This image demonstrates the variation in spectral data provided in both extract A and extract B for *C. glabrata* NCPF 8814 (red spectrum) echinocandin resistant, as m/z after 24 hours growth on SABC agar, when using sinapinic acid (SA) as a matrix in contrast with α-cyano-4-hydroxycinnamic acid (HCCA). In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = absorbance units, m/z = mass/charge ratio. (Author's own image)

3.3. Protein analysis by MALDI-TOF MS

3.3.1 Creating a profile for resistant isolates

Spectral records were created as detailed in Section 2.5.4 for selected isolates that exhibited *FKS*1 and *FKS*2 mutation-based resistance. This allowed the visual and statistical comparison of spectra to ascertain if differences between echinocandin resistant and echinocandin susceptible isolates was detectable solely by performing the standard methodology for organism identification. Main spectrum profiles (MSPs) were analysed in parallel within the same MALDI-TOF MS run to avoid inter-run variability, in terms of matrix composition and crystallisation (McComb *et al*., 2007), and also included in the routine analysis of yeast identification at the MRL. This provided a real-time comparison of known echinocandin resistant strains with isolates of *C. glabrata* of unknown susceptibility and allowed the demonstration of specificity of the created MSPs. No discrimination between resistant and susceptible isolates, with or without *FKS* mutations, was detected (Figure 12 and Table 14).

All the database matches present in Table 14, with the exception of the MSP for NCPF 8814 created as part of this study, were from isolates assumed to be echinocandin susceptible due to the lack of supporting information provided by the manufacturer. This suggests that any differences which may be present in spectral profiles between resistant and susceptible isolates of *C. glabrata* are undetectable using this type of data analysis protocol. This indicates that some form of manipulation to either the extraction protocol, the spectral acquisition methodology or the way in which the isolate is handled and prepared, prior to and during, extraction is required in order to further reveal the susceptibility profile of a given isolate.

In a direct comparison of MALDI-TOF MS spectra generated for *FKS*1 or *FKS*2 mutants, and those isolates not exhibiting echinocandin resistance, it can be demonstrated that there is little similarity in spectra collected from independent isolates when analysed concurrently (Figures 11-12). To investigate this further, the MALDI-TOF MS spectra data of isolates NCPF 8814 and NCPF 8745 were analysed using principle component analysis (PCA) as constructed by the Biotyper 3.0 software (Figure 13). By analysing correlated variables, in this case mass peaks present within the spectra, the dimensionality of the data is reduced allowing for a simpler visual interpretation of the dataset. Whilst it appears that there is a clear separation between the two analyses in components PC1 and PC2, in PC3 there is a level of spectral overlap indicating that some protein consistency between isolates is present, in particular between PC2 and PC3. There is a clear separation between the two isolates, but there is also evidence of variation amongst spectra from the same isolate between PC2 and PC3, suggesting potential issues with reproducibility if used for future spectral analysis. This reduces the discriminating power of MSPs created for these two isolates and indicates further that it is unlikely that resistant and susceptible isolates may be separated in this way.

Figure 14 presents a composite correlation index (CCI) or "heat-map" of spectral similarities between NCPF 8814 and NCPF 8745. This is another way of comparing all the spectra that form a given MSP. This appears to indicate that there is sufficient difference between the two MSP's for them to be separated (warm colours indicate similarity). However, the initial database interrogation shown in Figure 12 and Table 14 highlights that the spectral dissimilarity is insufficient or too incomplete to prevent cross matching with other database MSPs for *C. glabrata*.

Figure 15 demonstrates the differentiation between isolates exhibiting differing resistance mutations; *FKS1*: 61 and 75, *FKS2*; NCPF 8814 and 79. Although an element of similarity is indicated on PC3 of the isolates with an *FKS*2 mutation (Figure 15; green and yellow), the four isolates continue to demonstrate little comparability and once again, it is clearly demonstrated that there is little to no similarity in the spectra produced from isolates between or within the same *FKS* mutation. In fact, using the CCI analysis as shown in Figure 16, there appears to be more relative similarity between clinical isolate 61 and isolate NCPF 8814, which have different *FKS* mutations.

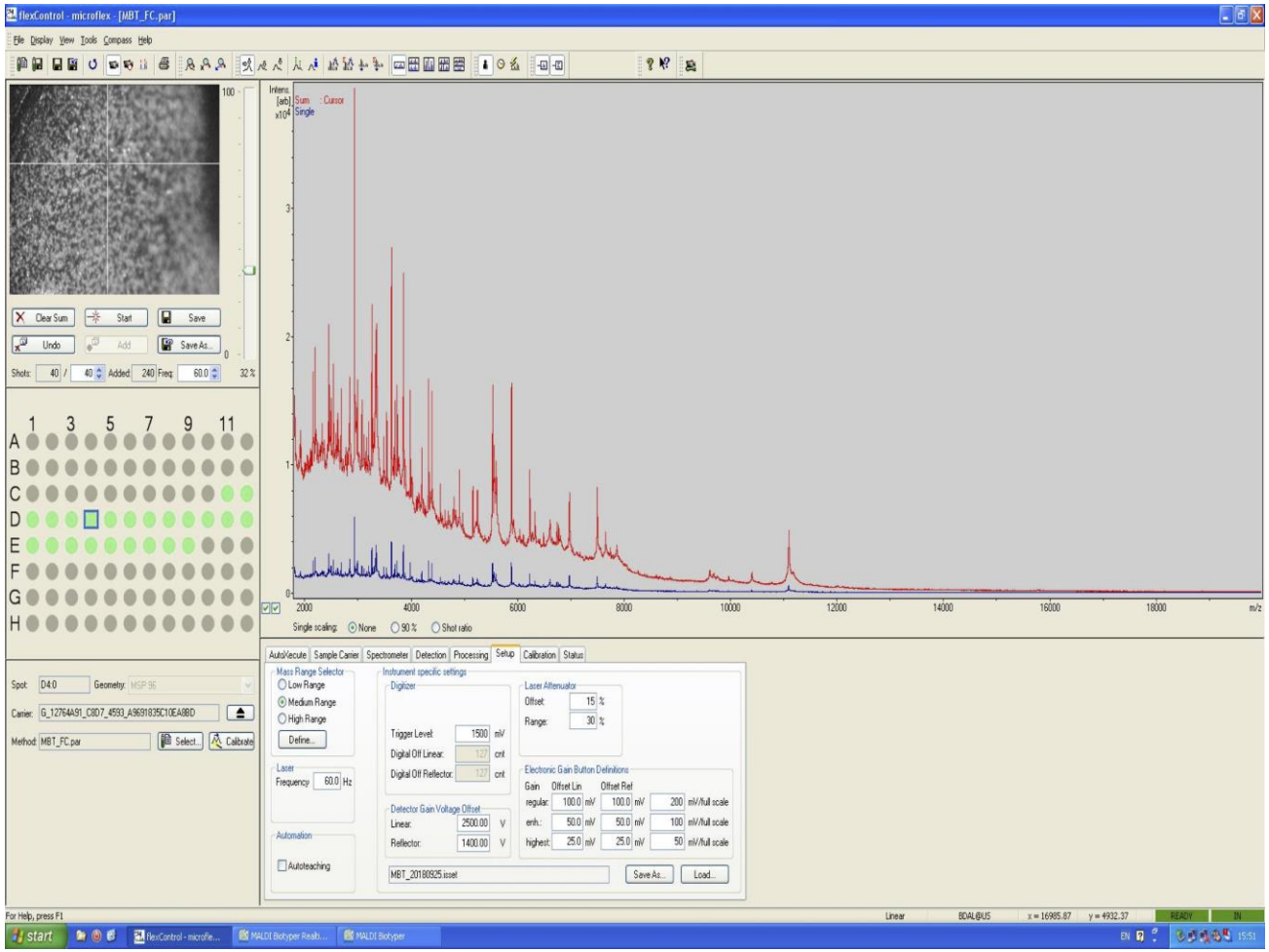

Figure 10. <u>An example MALDI-TOF MS spectrum for *C. glabrata* showing detection parameters of spectra acquisition</u>. This image displays the parameters for collection of spectral data used for routine yeast identification in the clinical laboratory as demonstrated for *C. glabrata* after 24 hours growth on SABC agar. The blue spectrum is a single acquisition and the red spectrum represents a sum of all spectra collected for this isolate (in total 24 replicate spectra). (Author's own image)

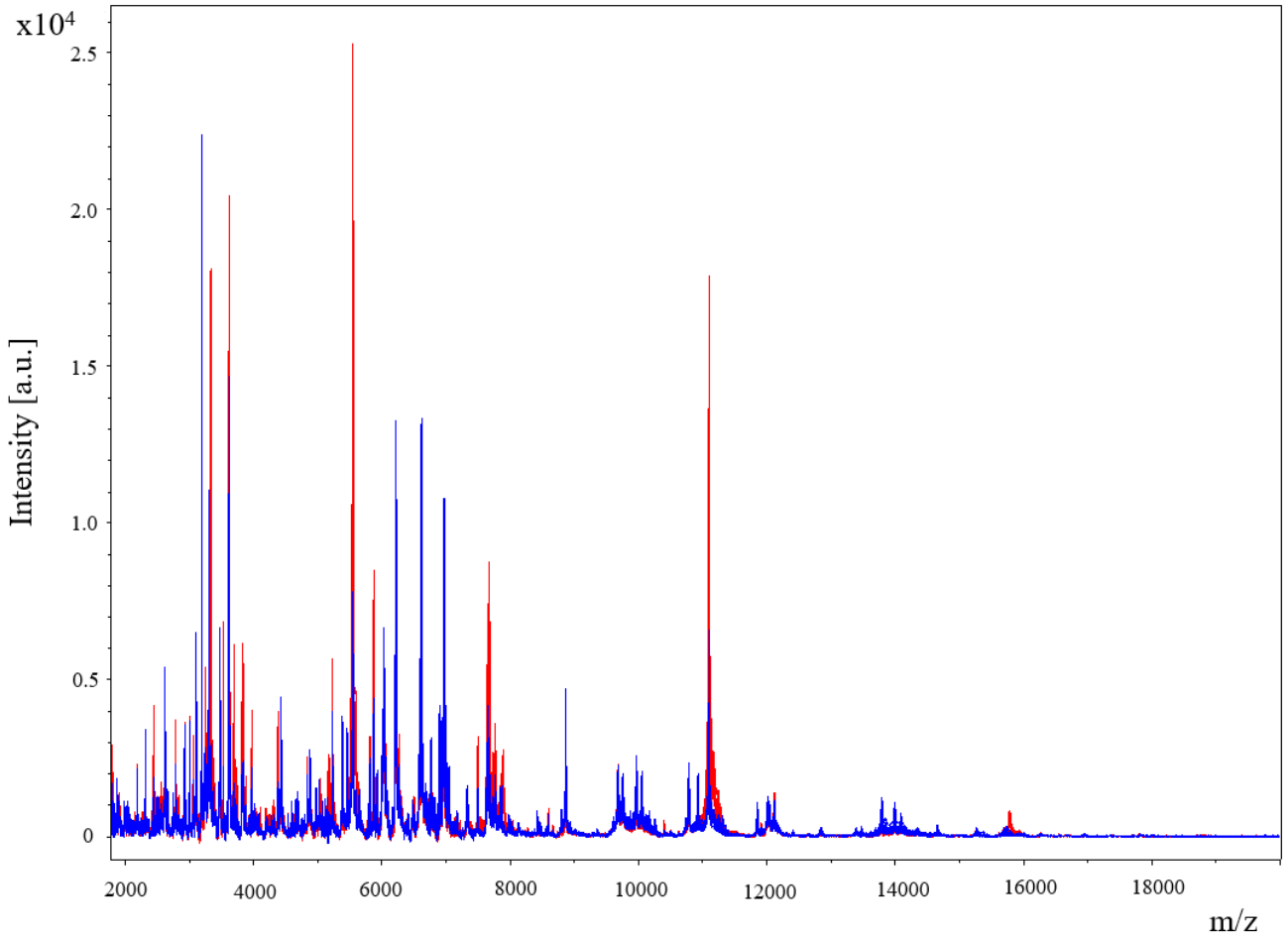

Figure 11. **<u>Comparative spectra generated for two isolates of *C. glabrata* by MALDI-TOF MS</u>.** This image portrays an example of two independent *C. glabrata* isolates which both exhibit *FKS*2 mutations after growth on SABC agar for 24 hours. Twenty-four replicate spectra for each isolate are overlaid to enable visual inspection of variation between the two isolates. Blue spectra represent NCPF 8814, red spectra represent clinical isolate 78. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary units, m/z = mass/charge ratio. (Author's own image)

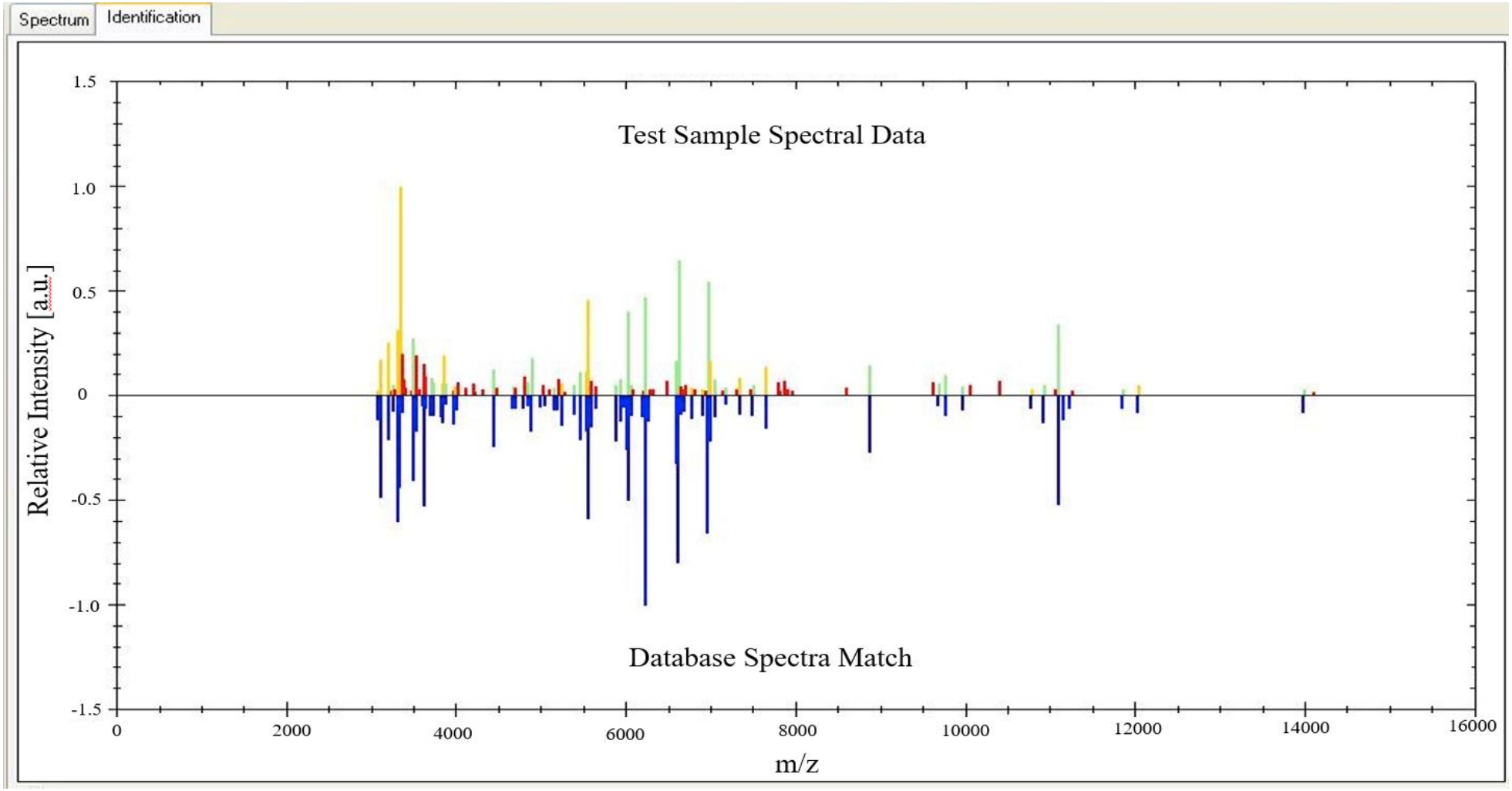

Figure 12. **Example database match for an uncharacterised clinical isolate of *C. glabrata*.** This image demonstrates the matching database record using Bruker's Biotyper 3.1 software for an uncharacterised clinical isolate of *C. glabrata* after growth on SABC agar for 24 hours. The top database matches for this isolate can be found in Table 14. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary unit, m/z = mass to charge ratio. (Author's own image)

Table 14. <u>MALDI-TOF MS database matches for an uncharacterised clinical isolate of *C. glabrata*</u>. This table shows the top ten MALDI-TOF database matches for a single clinical isolate of *C. glabrata* presented in Figure 12. The isolate was grown for 24 hours on SABC agar at 30°C. All the identifications and log scores returned represent acceptable results with the correct identification and log scores >2.000. The given Log Score is a logarithmic scale whereby 100% match is represented by 3.000. These data demonstrate the inability of MALDI-TOF MS to distinguish between echinocandin resistant and susceptible isolates of *C. glabrata*. The top two matches are for NCPF 8814, echinocandin resistant, and NCPF 8745, echinocandin susceptible. The clinical isolate was reported as echinocandin susceptible with an anidulafungin minimum inhibitory concentration (MIC) of 0.03 µg/mL.

Key: MIC = minimum inhibitory concentration (µg/mL), ANF= anidulafungin, CSP = caspofungin, MCF = micafungin, na = not available, Source = Culture collection reference, MRL = Mycology Reference Laboratory UK, CBS = Centraalbureau voor Schimmerlcultures The Netherlands.

Identification	Log Score	MIC (µg/mL) ANF/CSP/MCF	Source
***Candida glabrata* 8814 (S633P) MRL**	**2.364**	**4/6/1**	**MRL**
***Candida glabrata* 8745 MRL**	**2.315**	**na/<0.015/na**	**MRL**
***Candida glabrata* CBS 2663 CBS**	**2.300**	**na**	**CBS**
***Candida glabrata* CBS 8947 CBS**	**2.289**	**na**	**CBS**
***Candida glabrata* 10035463 101 USH**	**2.276**	**na**	**Unknown**
***Candida glabrata* CBS 858 CBS**	**2.155**	**na**	**CBS**
***Candida glabrata* CBS 863 CBS**	**2.123**	**na**	**CBS**
***Candida glabrata* CBS 862 CBS**	**2.117**	**na**	**CBS**
***Candida glabrata* 31 PSB**	**2.115**	**na**	**Unknown**
***Candida glabrata* CBS 858 CBS**	**2.094**	**na**	**CBS**

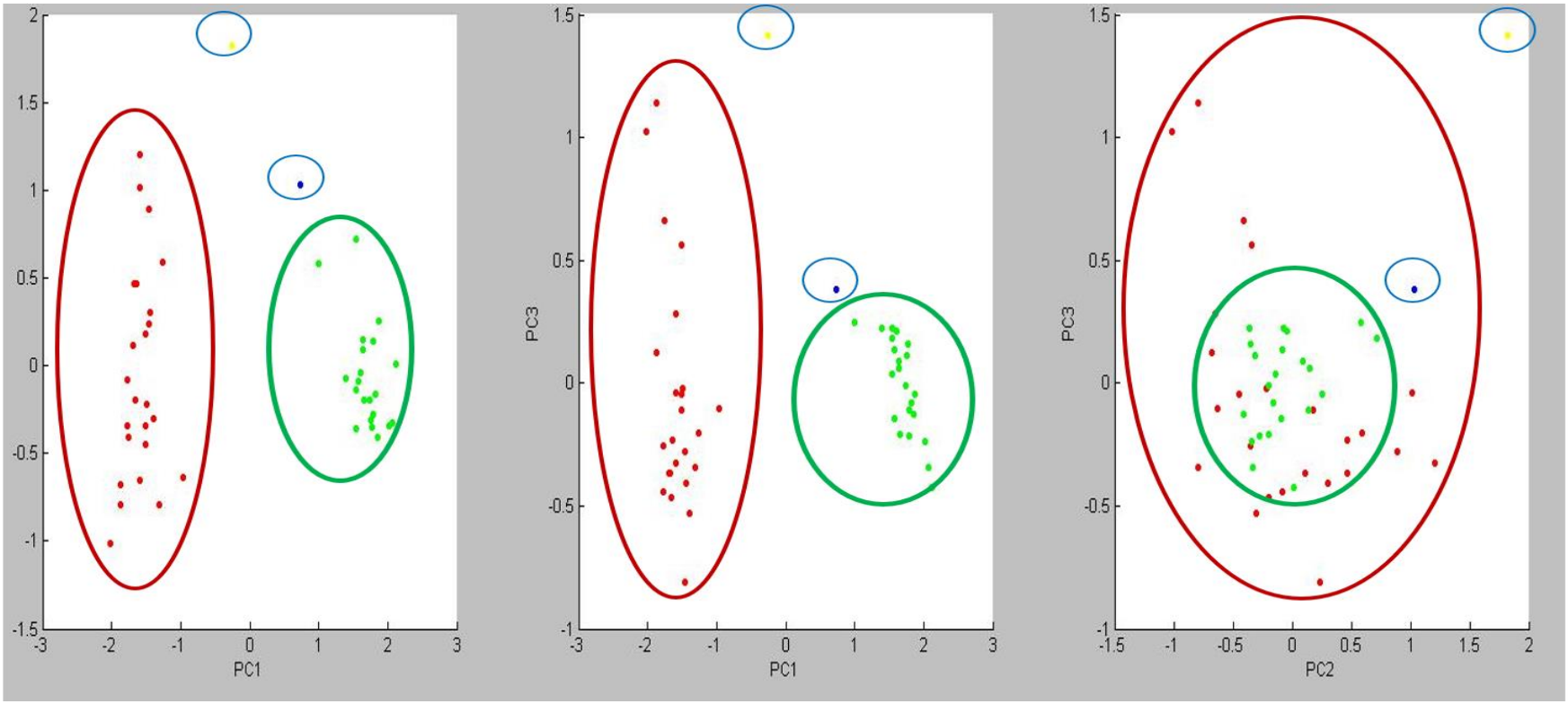

Figure 13. <u>A principal component analysis (PCA) of spectral data for NCPF 8745 and NCPF 8814.</u> A PCA for two sets of twenty-four representative spectra used for the creation of an MSP from NCPF 8814 (Red; echinocandin resistant) and NCPF 8745 (Green; echinocandin susceptible). Principal components (PC) 1,2 and 3 from the dataset are selected by Bruker's Biotyper 3.1 software. There is a clear separation between the two isolates, but there is also evidence of variation amongst spectra from the same isolate, suggesting potential issues with reproducibility if used for future spectral analysis. The blue and yellow dots (encircled in blue) represent a single bacterial control (Bacterial Test Standard, product code: 8255343; Bruker Daltonics GmBH, Bremen, Germany) which was included in this analysis to provide an indication of accuracy of the separation of principal components. (Author's own image)

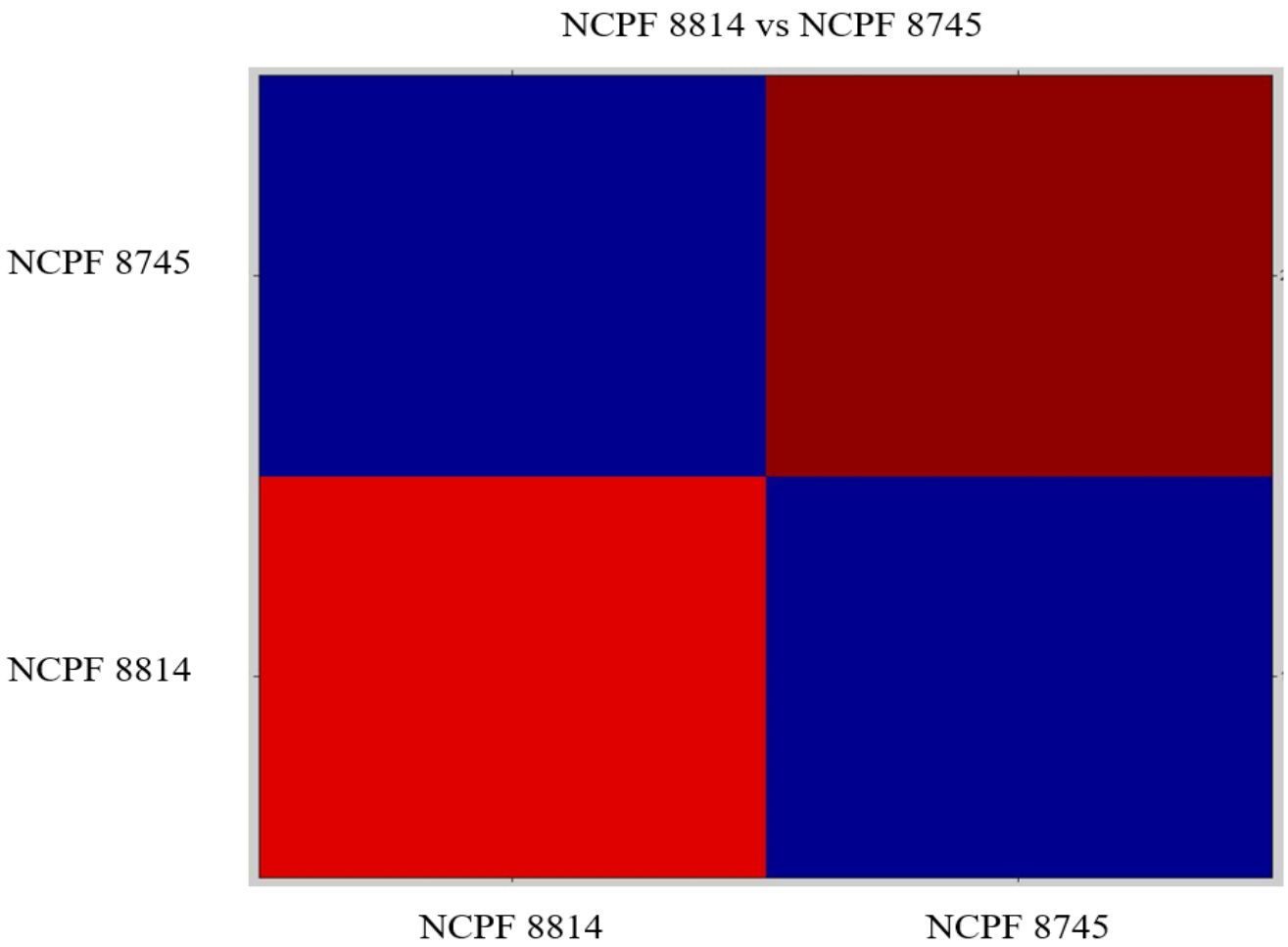

Figure 14. <u>A composite correlation index (CCI) to compare spectral data for NCPF 8745 and NCPF 8814.</u> A (CCI) heat map indicating relative comparability of spectra used to create MSPs for NCPF 8814 and NCPF 8745. Warm colours indicate similarity. Therefore, demonstrating that there should be a good level of differentiation between spectra from the two phenotypes. (Author's own image)

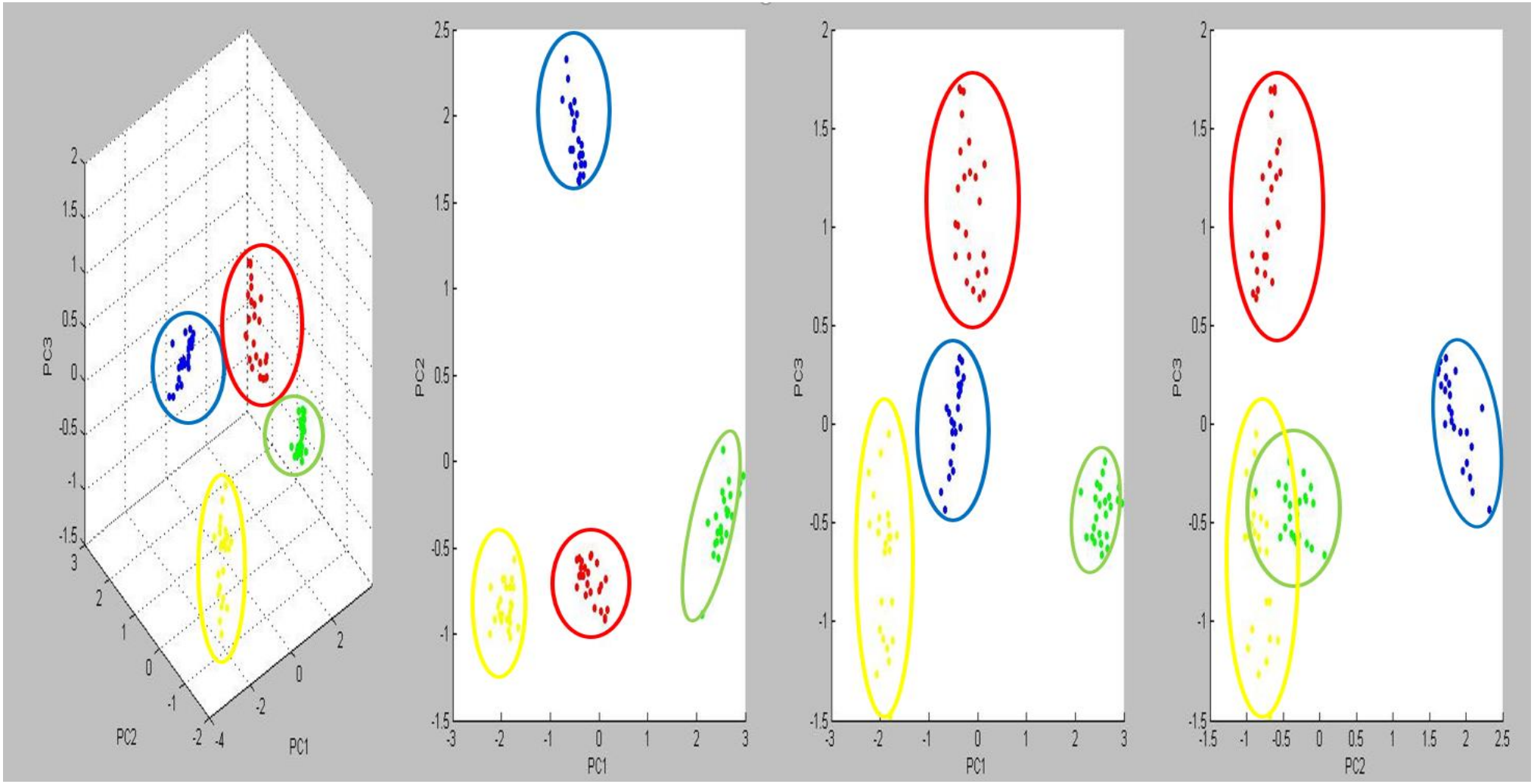

Figure 15. **<u>A principal component analysis (PCA) for four unrelated isolates of echinocandin resistant *C. glabrata*.</u>** A PCA constructed to show four unrelated isolates of *C. glabrata*. Two isolates with *FKS*1 mutations (blue and red; clinical isolates 61 and 75 respectively), and two isolates which exhibit *FKS*2 mutations (yellow and green; NCPF 8814 and clinical isolate 79, respectively). Principal components (PC) 1,2 and 3 from the dataset are selected by Bruker's Biotyper 3.1 software. (Author's own image)

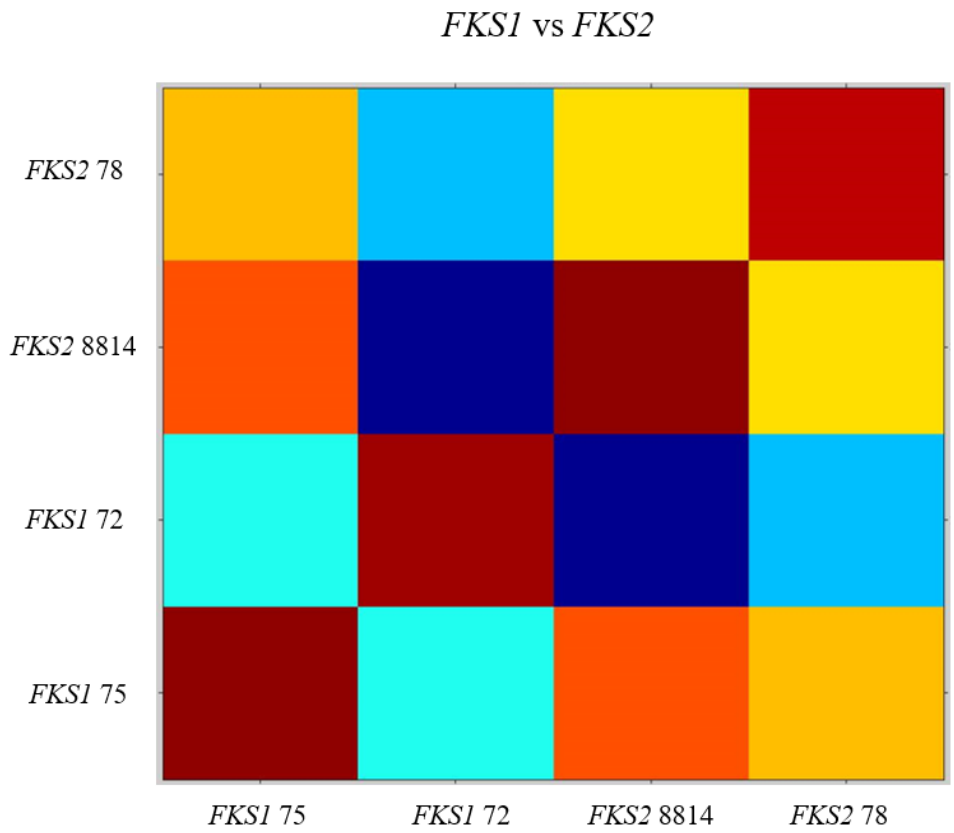

Figure 16. <u>A composite correlation index (CCI)to show the spectral relationship between four unrelated isolates of echinocandin resistant *C. glabrata*</u>. A CCI to indicate relatedness of spectra from isolates of *C. glabrata* with mutations in *FKS*1 (clinical isolates 72 and 75) and *FKS*2 (NCPF 8814 and clinical isolate 78). Warm colours represent similarity therefore, the dark red squares diagonally through the centre of the index represent a like for like comparison. (Author's own image)

3.3.2 The effect of growth in the presence of echinocandin on MALDI-TOF MS spectra

Figure 17 shows the MALDI-TOF MS spectra generated with NCPF 8814 following incubation using a standardised microbroth dilution susceptibility testing format (fully described in Section 2.2.2) for a period of 24 hours in the presence of a caspofungin serial concentration gradient (0.03-16 μg/mL). At and above MIC values considered resistant (>0.5 μg/mL), the pattern of peaks present in the spectral profile generated appears markedly different, particularly at values around 4 μg/mL or greater. The recorded MIC for NCPF 8814 is 6 μg/mL (Table 7). The nature of the spectra generated at 1 μg/mL and 2 μg/mL would be consistent with evidence of over activity, perhaps indicating some stress-activated response due to the presence of the echinocandin agent.

In order to explore the requirement for rapid testing in the clinical diagnostic laboratory, the next series of experiments focused on determining the minimum amount of time required from inoculation to the appearance of detectable levels of protein for spectra generation in the MALDI-TOF MS extract. Figure 18 shows the passage of time from 0-48 hours at a concentration of antifungal (CSP) equivalent to 1 μg/mL. Samples were taken at selected time points conducive with routine clinical laboratory working hours, between 1 to 48 hours incubation There was a reduction in the number of peaks detectable at 48 hours of growth. It was therefore determined that 24 hours were required for sufficient growth in the microtitre susceptibility format for a full spectral profile to be generated with NCPF 8814 during the presence of an antifungal. This can be seen in Figure 18. Whilst some mass peaks are present within 2 to 4 hours of growth, the amount of data available for analysis is greatly increased at 24 hours, whilst reduced at 48 hours.

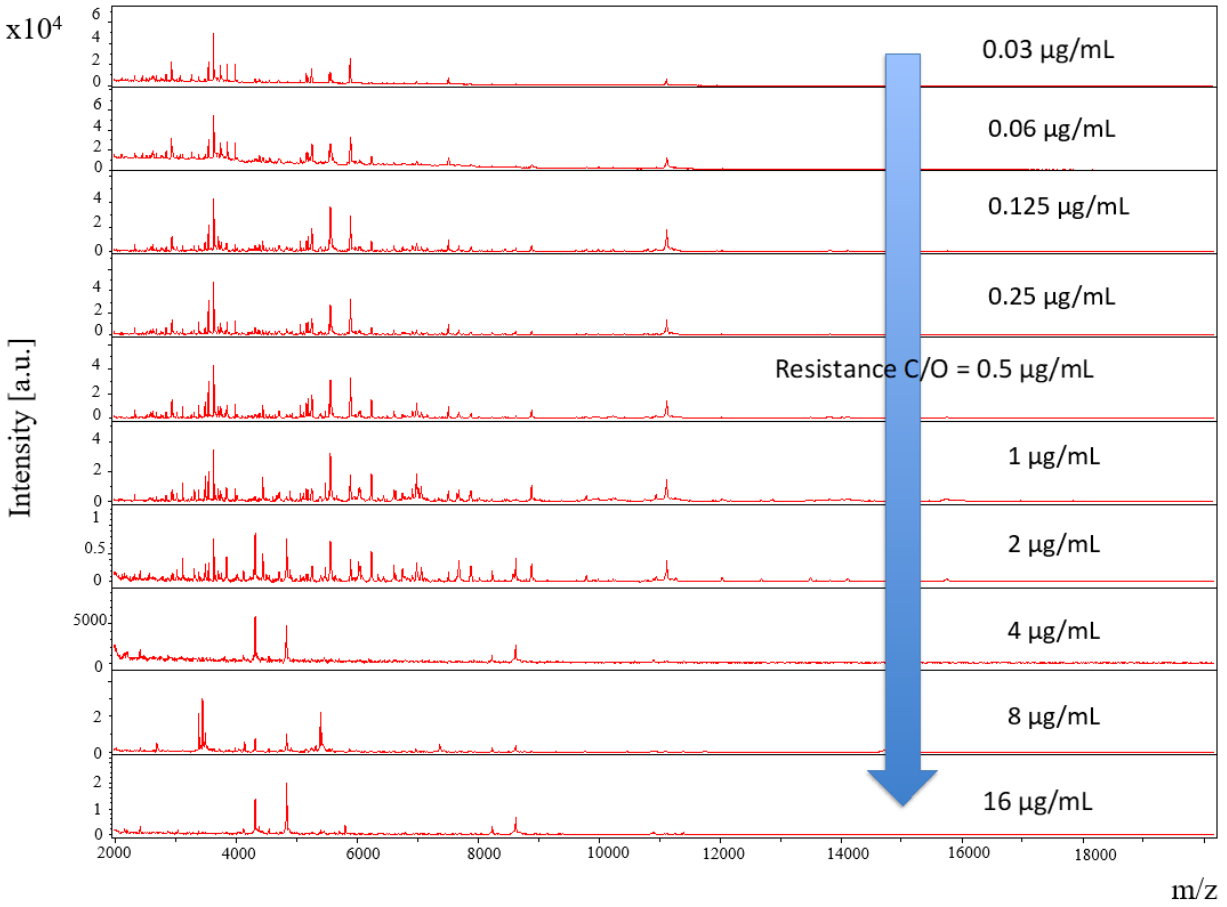

Figure 17. **<u>MALDI-TOF MS spectra generated for NCPF 8814 over a concentration gradient of caspofungin</u>.** A visual representation of spectra generated with NCPF 8814 incubated for 24 hours in RPMI liquid media in the presence serial concentration gradient of caspofungin (0.03-16 µg/mL). At MIC values considered resistant (>0.5 µg/mL), the pattern of spectral profile generated appears markedly different, especially at values greater than the MIC for this isolate (6 µg/mL). In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary units, m/z = mass/charge ratio. (Author's own image)

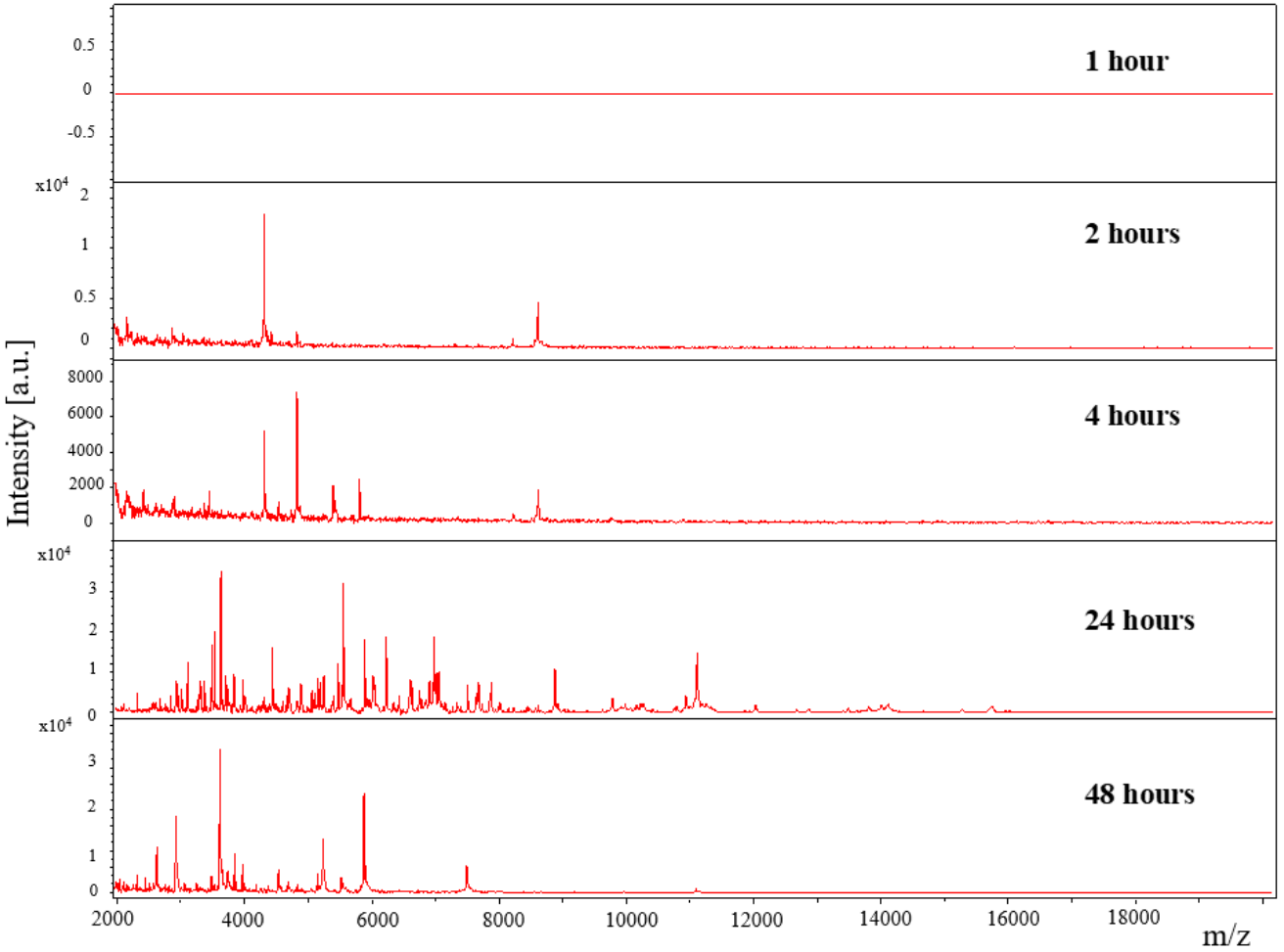

Figure 18. **<u>MALDI-TOF MS spectra generated at different time points during the incubation of NCPF 8814 in 1µg/mL caspofungin.</u>** This image shows the spectra generated at 1, 2, 4, 24 and 48-hour intervals with NCPF 8814 at a caspofungin concentration of 1 µg/mL in RPMI liquid media at 30°C. Demonstrating that in microbroth preparation, a 24-hour incubation results in the largest production of identifiable protein peak data, suggesting that this might be the most useful duration to detect peaks indicative of resistance. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary units, m/z = mass/charge ratio. (Author's own image)

3.3.3 The effect of increasing the fungal load and sample volume

The early work of this study looking at possible MALDI-TOF MS detection demonstrated that under the conditions used for routine susceptibility testing the volumes and quantity of biomass present was probably a prohibitive factor to the detection of visible spectra within an acceptable diagnostic timeframe (i.e. quicker than currently available methodologies) (Figure 18). Thus, an adaptation of the prescribed method was undertaken to include a larger initial culture volume for the MALDI-TOF MS extraction process to provide a greater quantity of final extracted protein. A desirable working volume of 10 mL was selected in order to provide adequate volumes for repetitious sampling and also to allow for an increase in initial fungal load. The methodology used to undertake these tests is fully described in Section 2.2.3. Initial growth experiments were performed to determine the optimum duration and method of incubation for visible cell growth. This included a comparison between growth in a stationary or a rotary position, with and without the presence of an antifungal. Where an echinocandin was included, CSP was used and two different concentrations were selected, a high concentration to indicate resistance (16 μg/mL) and a concentration that could mimic achievable *in vivo* levels (1 μg/mL). Growth was monitored over 24-48 hours. The results are shown in Table 15. The echinocandin resistant isolate (NCPF 8814) grew rapidly in the presence of CSP when the culture was rotated in an incubator at 30°C ± 2ºC, and within 24 hours confluent growth was evident. The echinocandin susceptible (NCPF 8745) isolate grew very poorly in the presence of 1 μg/mL after 48 hours.

Based on these results, further experimental cultures were grown in 10 mL glass tubes, placed on a rotary wheel and incubated at 30ºC ± 2ºC. Once inoculated, the cultures were incubated for 24 hours with the periodic removal of 100 μL volumes at selected time points. Each sample aliquot underwent a wash step using sterile distilled water to remove growth media from the

cells, followed by protein extraction as previously described (Section 2.5.2). As demonstrated in Table 15, growth of the echinocandin resistant isolate (NCPF 8814) was limited at 16 µg/mL almost to the point of prohibition until a time period of 48 hours, therefore all further tests were undertaken at a single concentration of 1 µg/mL of CSP. This concentration maximised the growth of organism whilst retaining the experimental procedure within a useful diagnostic timeframe.

Table 15. <u>Results of growth experiments for macro-broth cultures</u>. Growth of NCPF 8814 (resistant) and NCPF 8745 (susceptible) in 2 mL RPMI with and without caspofungin at 30°C in either a stationary (tube rack) format or on a fixed speed rotary wheel (20 rpm). These experiments were performed in triplicate with reproducible results.

Key: **Blank** = (no fungus, no antifungal - media sterility control), + = visible growth, - = no evidence of growth, +- = faint growth.

NCPF isolate	**Caspofungin µg/mL**	**Stationary incubation**		**Rotary incubation**	
		24 hours	**48 hours**	**24 hours**	**48 hours**
8814	**0**	++	++++	++++	++++
	1	+	++++	++++	++++
	16	-	+-	+-	+++
8745	**0**	-	++	+-	+
	1	-	+-	-	-
	16	-	-	-	-
Blank	**0**	-	-	-	-

The resultant MALDI-TOF MS spectra from growth after 24 hours stationary or rotary incubation at 30°C are shown in Figure 19. When analysed, the appearance of a triplet of peaks

at mass positions of 6227, 6627 and 6975 m/z in *FKS* mutant resistant isolates of *C. glabrata* during growth at concentrations of CSP above the MIC was demonstrated (Figure 19). These peaks were considered to be potential rapid visual identifiers for the protein-based detection of resistance and so were explored further.

Three further experimental replicates from the same isolates demonstrated the consistent presence of the triplet peaks within the spectra generated with NCPF 8814 (data not shown). Therefore, the experiment was repeated with a selection of clinical isolates from earlier in this study that could provide a representation of the anticipated variability in both *FKS* mutations and MIC distributions demonstrated in a clinical setting. Table 16 shows the isolates selected and the presence or absence of the triplet signature peak starting at the given MIC for an individual isolate and serial gradient concentrations above the MIC up to a final concentration of 16 μg/mL. Isolates were selected based upon *FKS* mutation and MIC value to demonstrate a full complement of variations in resistant or intermediate echinocandin susceptibility classification. This round of experiments was conducted on the chosen isolates four times to demonstrate reproducibility of peak pattern. Clinical isolate 67 demonstrated an MIC of 0.5 μg/mL, the cut off value for determination of resistance, however it does not present an *FKS* mutation and did not show the presence of the triplet signature. In addition, the triplet peak motif was undetectable in the spectra produced for isolate 71, which does exhibit an *FKS*1 mutation. These inconsistencies indicate that the triplet peak signature may not be an appropriate protein-based target to rapidly indicate echinocandin resistance in *C. glabrata* in the clinical diagnostic laboratory and further work on this was not attempted. Whilst these triplet peaks may represent some evidence of increase in abundance of proteins which may contribute to echinocandin resistance mechanisms in *C. glabrata*, that work falls outside the remit of the focus of this study.

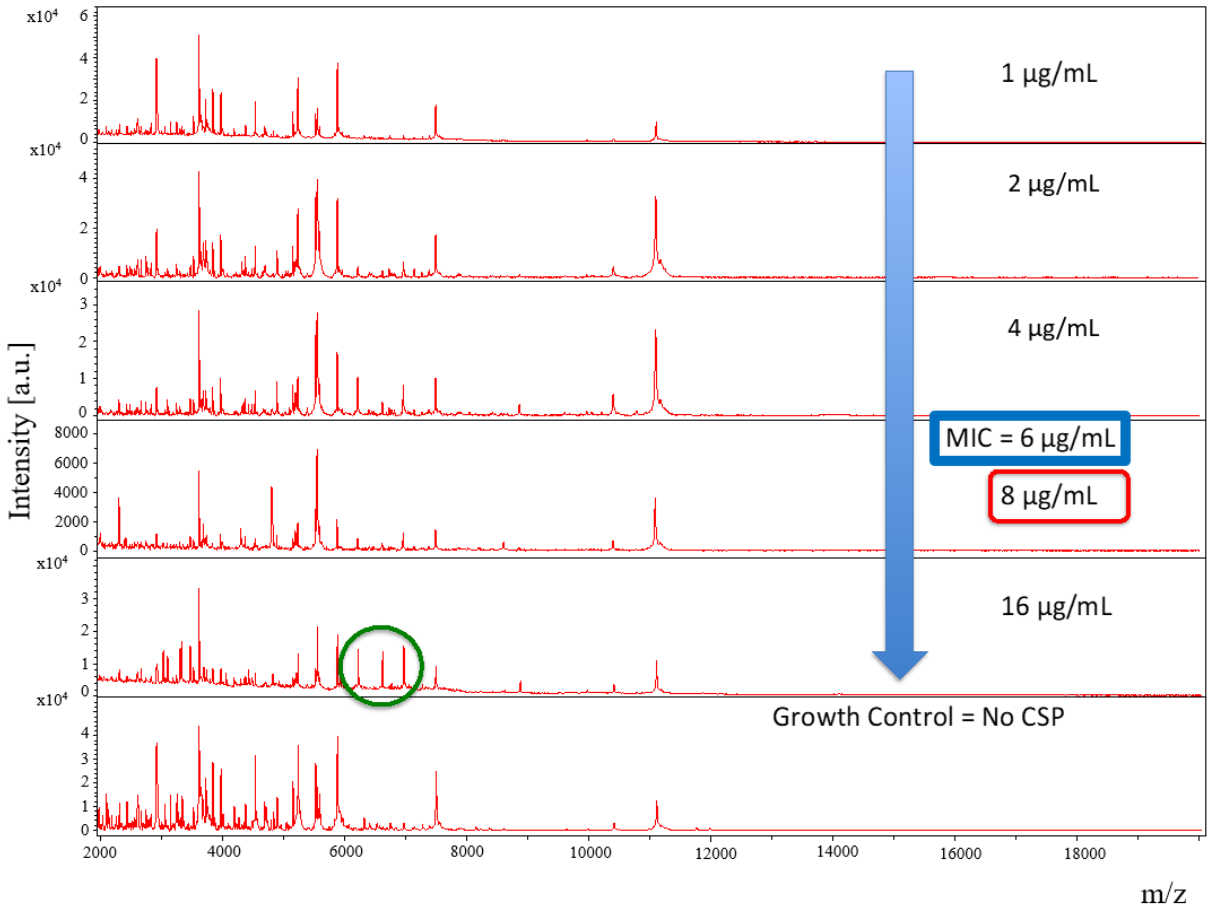

Figure 19. **<u>MALDI-TOF MS spectra for NCPF 8814 over a concentration gradient of caspofungin identifying a potential marker of resistance</u>.** The presence of a triplet of peaks in NCPF 8814 at 16 µg/µL (green circle) can be seen. This pattern is not present in the growth control (no antifungal) or sufficiently resolved at concentrations below the MIC (6 µg/mL) for this isolate following incubation at 30°C at 24 hours in RPMI liquid media in rotary phase (20 rpm). The spectrum generated at 8 µg/mL (red box) represents a poor recovery of proteins from the extraction of this sample. In mass spectrometry, M stands for mass and Z stands for charge (number of ions), this provides a scale of mass along the bottom of the spectrum. Z is almost always considered to be 1, the m/z value is therefore accepted as the mass value of the protein. Key: [a.u.] = arbitrary units, m/z = mass/charge ratio. (Author's own image)

Table 16. <u>The presence or absence of a potential marker of resistance as detected by MALDI-TOF MS in a selection of echinocandin resistant *C. glabrata* isolates</u>. Table depicting the presence of triplet proteins when selected study isolates were grown in the presence of caspofungin starting at concentrations equal to the MIC for each given isolate and each serial doubling dilution afterwards up to 16 µg/mL, as performed by macro-broth dilution and sampled after a period of 24 hours using a rotary incubation at 30ºC ± 2ºC. These experiments were conducted on the same isolates on four separate occasions.

Key: wt = wild type, m/z = mass peak value

Isolate (*FKS* mutation)	**Concentrations tested (µg/mL)**	**6227 m/z**	**6627 m/z**	**6975 m/z**
8814 (2)	4-16	✓	✓	✓
8919 (2)	16	✓	✓	✓
73 (2)	16	✓	✓	✓
67 (wt)	0.5-16	✗	✗	✗
72 (1)	16	✓	✓	✓
71 (1)	4-16	✗	✗	✗

4. DISCUSSION

With reference to the main study aim as laid out in Section 1.8, this study by adopted a methodological approach which employed the use of *Candida glabrata* and the echinocandin class of antifungal agents as a laboratory model of antifungal resistance. This study was designed in order to determine the viability of a bench-top genomic technology, pyrosequencing, and recently introduced proteomic platforms such as LC-MS/MS and MALDI-TOF MS for the detection of resistance in the clinical diagnostic laboratory.

The underpinning intention of this research was to enable the improvement of the detection of specific antifungal resistance markers within a clinically effective time frame in the diagnostic laboratory. When adopted as standard practice, such techniques would have the potential to positively influence therapeutic decisions and ultimately treatment outcomes.

Current methodologies for the detection of antifungal resistance in the clinical mycology laboratory involve susceptibility testing either by microbroth dilution or by E-test, as there is little capability for molecular methods in most routine mycology laboratories (Astvad *et al*., 2018; Klingspor *et al*., 2018; Lockhart *et al*., 2017; Pfaller *et al*, 2014a). Consequently, interpretative results are only available, and therefore actionable, no earlier than 24 hours post isolation from blood in cases of candidaemia. This means that realistically the minimum amount of time from sampling at the patient's bedside to reporting of results is a minimum of 48 hours in total (Mauri *et al*., 2017). Some guidance as to targeted therapy may be ascertained from rapid organism identification, and the introduction of rapid protein analysis by MALDI-TOF MS, has greatly improved the time required to return an organism identification (Curtoni *et al*., 2017; Idelevich, Grünastel and Becker, 2016; Fraser *et al*., 2016; Tran *et al*., 2015). However, the generation of fully interpretative susceptibility data still requires a minimum of 24 hours, even if susceptibility testing is performed directly from positive blood culture

isolation as suggested by Bordallo-Cardona *et al*. (2018). It should be noted that testing in such a way does not account for infection with more than one organism. This may have a negative effect on the testing in terms of interpretation of results which could lead to inaccurate and misleading clinical reporting, further adding critical time delays to the application of clinical management (Sante *et al*., 2019).

As suggested by Cendejas-Bueno, Romero-Gómez and Mingorance (2019), it is the integration of molecular assays into the routine workflow in clinical laboratories which would allow the genomic detection and profiling of BSI organisms, such as *C. glabrata* and *FKS* mutations that has the greatest potential to transform patient care.

This is particularly the case where the test in question can be performed directly on blood samples without the requirement for incubation and organism isolation. This could provide a further reduction in the time needed to report important therapeutic information to the clinician. Indeed, Idelevich, Grünastel and Becker (2016) suggested a methodology for the agents of candidaemia which resulted in the identification of the causative organism within 3.5 hours incubation on solid SABC medium, by using a centrifugation technique to concentrate cell mass. However, the biomass requirement for MALDI-TOF MS identification is considerably less than that required for current susceptibility testing methodologies (Klingspor *et al*., 2018; Lockhart *et al*., 2017; CLSI, 2017a; CLSI, 2017b; Fraser *et al*., 2016).

As outlined in Objective 1 of the study aim, the approach to genetic detection of resistance markers using pyrosequencing demonstrated in this thesis will provide the ability for the clinical mycology laboratory to report specific resistance mutations to a single class of antifungal agents for *C. glabrata* within 4 hours of first isolation. If combined with a rapid recovery methodology, such as that suggested by Idelevich, Grünastel and Becker (2016), there exists the potential for actionable, targeted therapeutic decisions within 7-8 hours, despite being highly specific to a single organism and resistance mutation.

4.1 Analysis of *FKS* mutation detection as a predictor of resistance

The 0.55% prevalence of echinocandin resistance in *C. glabrata* demonstrated in the UK during the period of this study correlates well with the reported levels of echinocandin resistance seen in similar retrospective studies conducted in other European countries and China (Astvad *et al*., 2018; Mencarini *et al*., 2018; Hou *et al*., 2017; Shields, Nguyen and Clancy, 2015; Marcos-Zambrano *et al*., 2014). It also appears consistent with the re-evaluated *in vitro* data generated at PHE MRL prior to the specific timeframe of this study (Fraser *et al*., 2019a).

Eleven isolates (clinical isolates 61, 71-76, 78-81) demonstrated MICs which would be classified as resistant for two of the tested echinocandin agents. Two further isolates (clinical isolates 67 and 77) demonstrated evidence of a distributed pattern of resistance, with resistant MICs demonstrated for CSP, intermediate MICs for ANF whilst maintaining susceptibility to MCF. The remaining two isolates were only tested against CSP (isolates 68 and 70). Of particular note, 6/15 (40%) of the isolates originated from bloodstream infections, and 10/15 (66%) isolates were referred from different centres within the geographical region of London. None of the isolates in this study represented repeat isolations from the same patient, although 3/15 (20%) did originate at the same London centre. However, they were isolated 2 and 6 months apart, therefore reducing the likelihood of a specific outbreak scenario at the referral institution (Fraser *et al*., 2019a).

The present study showed that 6/15 (40%) of the resistant clinical isolates demonstrated a mutation in *FKS1*, with 6/6 (100%) of the detected mutations representing an amino acid substitution of serine for proline at position 629 (S629P; Table 10). For *FKS2*, 4/15 (26.7%) of

resistant isolates showed a definitive mutation, with once more 4/4 (100%) representing a serine to proline substitution but at position 663 (S663P; Table 10).

The presence of a single mutation in either *FKS1* or *FKS2* was sufficient to confer resistance to all echinocandin agents in accordance with previous reports (Lackner *et al*., 2014; Katiyar *et al*., 2012; Niimi *et al*., 2012; Zimbeck *et al*., 2010).

Of those isolates demonstrating evidence of phenotypic resistance, 4/15 (26.7%) did not apparently possess a mutation in either *FKS1* or *FKS2* (clinical isolates 67, 68, 70 and 77). However, all 4 isolates demonstrated MIC values that bordered the cut-off (0.25 – 0.5 μg/mL) and would be phenotypically classified as intermediate. Generally, when the MIC value was at least two doubling dilutions greater than the susceptibility cut-off value, there was an associated *FKS* mutation present. A single exception to this rule was demonstrated with clinical isolate 71 where MICs to ANF and CSP were within 2 doubling dilutions from the break point value for susceptibility but an *FKS1* mutation was detected (Table 7 and Table 9).

The pyrosequencing assay to detect *FKS* mutations developed during this study provides a platform that has the potential to reduce the minimum detection time for a known resistance mutation to 4 hours post-isolation, and many clinical laboratories may already have access to the platform without further expenditure (Moore *et al*., 2016; Borman *et al*., 2010). Whilst this study used freshly sub-cultured organisms from referred clinical isolates, it would be possible to perform the pyrosequencing assay directly on isolates at the point of receipt or isolation, with studies suggesting the use of pyrosequencing directly from blood cultures (Fraser *et al*., 2016; Moore *et al*., 2016; McCann *et al*., 2015; Borman *et al*., 2010). In fact, one study found >90% success rates in the identification of bacterial species in patients being investigated for bloodstream infections in this way (Moore *et al*., 2016). There is therefore the suggestion that the possibility of performing an assay to detect mutations in *FKS* without the need for secondary sub-culture or incubation of further tests to determine susceptibility exists (Moore

et al., 2016; McCann *et al*., 2015), but this would require further optimisation and validation. This serves to highlight the potential for the use of pyrosequencing as a rapid molecular screening procedure or empiric antifungal triage service for all isolates of *C. glabrata* referred to the reference laboratory, or in centres were emergent resistance has been demonstrated. This would significantly reduce the time and potential financial impact to clinical centres from the inappropriate use of antifungal regimes, within 24 hours (Buehler *et al*, 2016; Perez *et al*., 2013), and provide a suitable alternative approach to detection of susceptibility demonstrated by many susceptibility test protocols (Pfaller *et al*., 2014a; Arendrup *et al*., 2010).

It was repeatedly difficult to ascertain a high confidence sequence (>99% coverage/identity) for *FKS2* with two of the clinical isolates (71 and 72). This could be due to multiple factors, including short primer length, or quality of the initial PCR amplification products. Further investigation of clinical isolates 71 and 72 demonstrated that they were unrelated in terms of site of isolation and geographical region of referral location (Table 1). It is reported that the suggested mutation in *FKS2*, S663F representing a substitution of serine for proline at hot spot position 663, has previously been associated with resistance (Pham *et al*., 2014b). If it is present in these isolates, the simultaneous presence of a mutation in *FKS1* (S629P) means that a second mutation in *FKS2* cannot be inferred purely from phenotypic observation alone. These isolates would be suitable candidates for further work, which could utilise NGS or WGS approaches, to ascertain their full genomic background and provide a more complete understanding of the exact nature of the genetic resistance mechanisms they may possess. However, this is beyond the remit and objectives of the current study.

The variability and inconsistency in the detection of mutations in *FKS*1 and *FKS*2, even in this relatively small subset of isolates, suggests that the detection of such mutations may not be the most reliable marker alone for the detection of resistance to echinocandin agents, and that the

role of susceptibility testing is still paramount to determining the appropriate selection of antifungal agents for treatment of infection.

The suitability of the selected primers for amplification of the region of interest and the effectiveness of the 3' redundancies introduced to avoid cross amplification between the two near-identical regions was clearly demonstrated. Whilst it was difficult to ascertain a high confident sequence read (>99% coverage/identity), most likely due to the short primer lengths, the amplified products and their database matches were sufficiently variable to deduce that neither primer caused cross amplification (Section 4.2).

The pyrosequencing of the *FKS*2 mutation region generated many indeterminate results suggesting the assay may not be appropriate for this region, or that the primers designed for this study may not be fit for purpose. This could be due to a redundancy within the PCR reaction, or due to the initial quantity of gene copies present. It has been previously demonstrated that the efficiency of the pyrosequencing method is optimised over a region of about 30-35 bases (Borman *et al.*, 2010; Borman *et al*., 2008). In order to capture all of the *FKS* hot spots, this has to be extended to 45 bases and it is possible that the internal chemistry of the assay becomes too unreliable at this point resulting in low score matches (Gharizadeh *et al.*, 2006). Homology scores less than 100% must be treated with caution when a single base alteration can result in the mutation of the gene, especially if it occurs within a hot spot region. Detection of *FKS* hot spot mutations can provide an explanation for the presence of resistance in an isolate but based upon the data presented here, it is not a sufficient indicator alone to determine antifungal treatment regimes.

It has previously been demonstrated that the efficiency of pyrosequencing is optimised over a region of 30-35 bases (Borman *et al*., 2010), but in order to capture all possible *FKS* hot spot mutation combinations, the assay used for this study was extended to read 45 bases. It is highly probable that the internal chemistry of the assay, in particular the effectiveness of the enzymes

present in the reagent mix becomes too unreliable at extended strand lengths and it was this which resulted in the low score matches observed with some *FKS2* sequences. Sequence homology scores less than 100% must be treated with caution when a single base pair alteration can result in the mutation of the gene, especially if it occurs within a hot spot region.

In agreement with published literature, this work has confirmed the use of *FKS* mutation detection as a useful tool in the diagnostic laboratory identification of echinocandin resistance. All isolates that demonstrated resistant MICs with two or more echinocandin agents had identifiable *FKS* hot spot mutations as detected by pyrosequencing. However, the demonstrated variability in successful detection of mutations in *FKS1* and *FKS2* detected by pyrosequencing, even at hot spot locations, suggests that it may not be the most reliable marker or methodology on its own for the detection of resistance to echinocandin agents, contrary to the suggestions by Shields *et al*. (2012).

A recent US study also found that phenotypic non-susceptibility, in particular with MCF, could be demonstrated in the absence of detectable *FKS* mutations (McCarty *et al*., 2018). Four isolates from the current work that did not have *FKS* mutations (isolates 67, 68, 69 and 77), phenotypically demonstrated MICs that would be considered intermediate or non-susceptible to CSP. As such the echinocandin antifungal agents would not be recommended as therapeutic options without further testing. This provides additional support for the continued role of traditional susceptibility testing in the determination of appropriate antifungal agent selection, as well as the continued use of ANF or MCF as indicators of true echinocandin resistance (Fraser *et al*., 2019a; CLSI, 2017b; Shields *et al*., 2013). The review of candidaemia in Latin America undertaken by da Matta *et al*. (2017) reported that echinocandin resistance had been detected only in *C. glabrata*, with the exception of a single study conducted in Argentina, which cited CSP resistance in *C. parapsilosis*. No single survey had confirmed the resistant phenotype using molecular methods, perhaps suggesting there is not the physical capability, or

no determined clinical need, to detect genomic markers of resistance to echinocandins at centres in Latin America. These data could also provide some further evidence that smaller clades of *Candida* spp., which only exhibit a phenotypic or non-*FKS* based genomic resistance, do exist (McCarty *et al*., 2018; Shields, Nguyen and Clancy, 2015; Vallabhaneni *et al*., 2015; Shields *et al*., 2013).

It is important to highlight that part of the function of a specialist reference laboratory is to undertake the investigation of unusual organisms and provide support for regional laboratories without specialist expertise. Therefore, data generated at the PHE MRL are often skewed towards those isolates that do not fit the trend, are difficult to interpret, or require confirmatory testing (Borman *et al*., 2012). Even so, the MIC data collected from testing using E-test for CSP and MCF, and CLSI for ANF prior to the *FKS* mutation detection trial, demonstrated a low echinocandin resistance rate of around 0.9-1.5% (Fraser *et al*., 2019a). With the increase in commercially available microbroth dilution systems which include all three currently available echinocandin agents (e.g. Sensititre Yeast One™, Thermo Fisher), more laboratories are moving towards in-house testing and it is important that laboratories using these systems are aware of potential difficulties in the interpretation of CSP MIC values and the potential clinical consequences (Aigner *et al*., 2017; Alfouzan *et al*., 2017; Pfaller *et al*., 2012c). When testing CSP, results may be reported as intermediate or resistant. However, CLSI (2017b) recommend that confirmatory testing is performed using either ANF or MCF, DNA analysis to confirm *FKS* hot spot mutation or by confirmatory testing at a reference laboratory. Regardless of an isolate's given CSP MIC result, the recommendation states that if either of the first two criteria is fulfilled, echinocandin resistance across the full range of available agents is confirmed and should be reported.

4.2 Re-evaluation of MIC data in the context of changing laboratory protocols

In total from 2003 to 2018, 7,225 clinical isolates of *C. glabrata* were tested for echinocandin susceptibility at the PHE MRL. Between 2003 and 2016 there was a considerable shift in MIC values reported for *C. glabrata* when tested against CSP (Fraser *et al*., 2019a). This was a direct acknowledgement that species-specific breakpoints were required (CLSI, 2017a; CLSI, 2017b) and as reported by Espinel-Ingroff *et al*. (2013), and that CLSI testing of CSP is now considered too variable between laboratories to reliably indicate resistance. As reviewed by Fraser *et al*. (2019a), MIC values generated by CLSI methodology for echinocandin testing at the PHE MRL from 2003 to 2013 indicated a prevalence of resistance ranging from 0.3- 7.9%, with a mean of 3.4%. The introduction of the use of ANF as a sentinel echinocandin to indicate susceptibility to the whole class of agents, and E-test for specific CSP testing, resulted in a shift in prevalence range to 0.9-2.7% with a mean of 1.5%. As testing was optimised and harmonised during the period of this study, the evidence presented between 2015 and 2018, indicates that the prevalence of echinocandin resistance in clinical isolates of *C. glabrata* in the UK continues to remain very low at 0.55%. This time period included the use of *FKS* mutation detection alongside microbroth dilution and E-test susceptibility testing (Table 7). As previously stated, these data correlate well with reported prevalence from studies conducted in other European countries (Astvad *et al*., 2018; Mencarini *et al*., 2018; Marcos-Zambrano *et al*., 2014) and China (Hou *et al*., 2017), and appears consistent with the re-evaluated *in vitro* data generated at PHE MRL prior to the specific timeframe of this study (Fraser *et al*., 2019a).

It is also important to note that a few discrepancies were detected with the use of culture collection isolates and this raises some important issues. Of those NCPF isolates selected for use in this study, 3/5 (60%) examples of echinocandin resistant *C. glabrata* were found to be

echinocandin susceptible upon revival (Table 6 and Table 8). None of these isolates exhibit any detectable *FKS* mutations using the methods adopted as part of this work. This could be an indication that resistance mechanisms acquired in the presence of antifungal treatment are lost during storage and revival, as previously described in methicillin-resistant strains of *Staphylococcus aureus* (van Griethuysen *et al*., 2005) and *Helicobacter pylori* (Henriksen *et al*., 2004).

Early studies had suggested that an S633P mutation in Fks2p did not result in altered fitness or responsiveness in an *in vivo* model of infection using the larval model *Galleria mellonella* (Borghi *et al*., 2014). It is possible that the loss of the *FKS* mutation may be related to a consequential fitness cost to *C. glabrata* as documented with *FKS2* (Bordallo-Cardona *et al*., 2017). Indeed, Imbert *et al*. (2016) demonstrated that the removal of an echinocandin during the treatment of candidemia with *C. glabrata* in a single patient resulted in the loss of the *FKS* mutation from subsequently identified clonal isolates. They concluded that this was evidence of the potential fitness cost that a mutation in *FKS* conferred upon an organism.

Alternatively, as previously discussed in this thesis, the discrepant results between storage and revival could be artefacts of alterations in the interpretation and breakpoint ranges between the commencement of testing in 2003, and those adopted as part of this study (Fraser *et al*., 2019a). Indeed, NCPF 8714 and 8715 were added to the collection in 2003 however, no susceptibility data from the original accession of these isolates could be located. This would suggest that these isolates represent earlier misinterpretation, and this serves to highlight the importance of validating culture collection isolates prior to their use in research, as well as emphasising the responsibility a culture collection has in maintaining and supplying accurate data for the organisms it holds (Smith, 2012). This includes the requirement for periodic revival and re-assessment of collection isolates with consideration for new research, such as the re-assessment and correlation of identification using MALDI-TOF MS (Lima-Neto *et al*., 2014), and updating

records to include developing taxonomy (Forti *et al*., 2016) or advancements in knowledge and technology (Fraser *et al*., 2019a; Borman *et al*., 2006; Brenner *et al*., 1995).

There is a well-documented paradoxical growth effect seen with some *Candida* species when performing susceptibility testing by microbroth dilution against the echinocandin agents, as reviewed by Wagener and Loiko (2017). This is demonstrated by the appearance of an increase in biomass growth at concentrations greater than the MIC, and therefore may be responsible for the reporting of falsely elevated MIC values. None of the susceptibility testing undertaken as part of this thesis presented such paradoxical growth effects, and this is in concordance with reports suggesting that this phenomenon has not been reported with *C. glabrata* and CLSI methodology (Shields *et al*., 2011; Chamilos *et al*., 2007). There is, however, a report of a small population of isolates demonstrating a paradoxical growth effect at high echinocandin levels using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology for susceptibility testing and this should be considered when interpreting results using this adapted methodology (Marcos-Zambrano *et al*., 2016b).

4.3 The use of proteomic approaches for the detection of resistance

As Otto, Becher and Schmidt (2014) suggest, advances in quantitative proteomics have led to a paradoxical situation whereby more techniques and software are available for the detection and analysis of proteomes but choosing the right technique to answer a specific scientific question is increasingly more difficult. There exists a need to fulfil basic scientific requirements whilst remaining within economic constraints for expenditure per dataset, and this is also true when applied to systems being considered for introduction into laboratory diagnostics. Issues of economics, time and data output are essential qualifiers for the successful implementation and uptake of new techniques or methodologies (Tran *et al*., 2015; Okeke, *et al*., 2011).

Developing technological approaches which use MALDI-TOF MS platforms have demonstrated the potential for the detection of resistance mechanisms in bacteria. Particularly, bacteria that present significant clinical challenges such as *Acinetobacter baumannii* (Dortet *et al*., 2018) and the Enterobacteriaceae (Figueroa-Espinosa *et al*., 2019), and developments have extended to *Candida* spp. (Delavy *et al*., 2019). There also exists the potential for the detection of specific antimicrobial resistance mechanisms such as demonstrated with carbapenemases (Vogne *et al*., 2014). The detection of proteins that have a role in the resistance of bacteria to colistin, one of the remaining agents active against multidrug resistant bacteria, has been demonstrated (Sun *et al*., 2017).

However, the financial constraints of acquiring the platform, coupled with time limitations, whereby a minimum incubation time of 6 hours post isolation is required prior to a result being reported (Vatanshenassan *et al*., 2018; Vella *et al*., 2017), suggests there is scope for the application of alternative approaches to better inform and improve the power of data generated by MALDI-TOF MS.

4.3.1 LC-MS/MS in the clinical diagnostic mycology laboratory

As detailed in Objective 2, this study has used LC-MS/MS as a sensitive and specific technique which has been used to identify potential protein targets for the detection of echinocandin resistance in *C. glabrata.* In doing so, this study has also offered a comparison of two different proteomic methods which should generate similar or translatable data for the detection of proteins which may relate to a specific resistance pattern in a single organism.

LC-MS/MS generates a large dataset of predicted proteins based upon peptide fragments detected in each sample. However, a significant financial burden per sample means that it is essential to be certain that the output provides the data necessary to move a project or clinical

decision forward (Zhao *et al*., 2019). The time required for manual analysis of these datasets is also a crucial factor to consider and is a large contraindication for its use in rapid clinical diagnostics in microbiology. However, LC-MS/MS is used extensively across the bioscience disciplines for activities as varied as the monitoring of therapeutic agents (Ndolo *et al*., 2016; Decosterd *et al*., 2010; Oellerich *et al*., 2004), biochemical metabolites and hormones (Morote *et al*., 2018; Welsh *et al*., 2017) and illicit drugs (Lendoiro *et al*., 2017; Miller *et al*., 2008), as well as the identification of microorganisms direct from blood culture bottles (Berendsen *et al*., 2017). Computer software packages can only organise and highlight data of potential interest to a point within the design and remit of each package, therefore the role of the researcher remains essential in the critical analysis of each data set. So, whilst it remains a powerful tool for the research and development of specific diagnostic tests, its role in clinical diagnostic microbiology is limited, unlike MALDI-TOF MS which has successfully developed into an essential diagnostic tool in clinical microbiology.

The greatest value to the clinical microbiology laboratory of LC-MS/MS is in its ability to provide the identification of specific proteins which could be used as targets to detect resistance using MALDI-TOF MS or alternative detection methods. There is an inherent expectation that proteins extracted for either methodology from the same sample would be consistent to allow the cross-detection necessary between methodologies. Also, the common appearance, or matching, of proteins by both methodologies would more likely indicate potentially significant markers of resistance/susceptibility, more so than if preferentially isolated in only one of the extraction protocols used.

This study has demonstrated considerable differences in the complexity of the two extraction protocols required for each method. For instance, whilst the MALDI-TOF MS extraction protocol (described in Section 2.5) can be performed within 10 minutes, and uses relatively simple chemical components such as water, ethanol, trifluoracetic acid and acetonitrile, the

methodology employed for LC-MS/MS protein extraction (Appendix III) requires the use of a large array of detergents, protease inhibitors and alkylating agents (iodoacetamide) and includes an over-night incubation period. This complex methodology serves to further separate the two techniques in terms of utility in the clinical diagnostic laboratory and re-affirms the use of LC-MS/MS as a tool of discovery for the facilitation and development of application technologies, as attempted with this study under Objective 2.

This effectively rules out LC-MS/MS as a diagnostic detector of proteome alterations on its own in the clinical mycology laboratory despite studies demonstrating the success of LC-MS/MS as a tool for bacterial identification (Berendsen *et al*., 2017). In addition, the outsourcing of work beyond the clinical laboratory can be very expensive and would ultimately result in further time delay to the reporting of clinical results. It should be noted that the limitations observed by the author for the use of LC-MS/MS in the clinical diagnostic laboratory is based on the single analysis of two isolates of *C. glabrata*. The analysis of a larger panel of representative examples of echinocandin resistant and susceptible isolates of *C. glabrata* might elucidate patterns of proteomic variation that may prove useful in the further development of the laboratory detection of resistance.

This study demonstrated little to no observable correlation between the two methodologies in terms of the generation and comparability of data. There was no evidence that proteins predicted from the LC-MS/MS prepared extraction were detectable by MALDI-TOF MS (Figure 9; Section 3.2.2 – Section 3.2.4). Therefore, it would seem that, at least from a clinical perspective, there is little value in directly comparing data generated by LC-MS/MS to that of MALDI-TOF MS generated data.

The recovery of proteins from the extraction processes used in this study appeared to be most productive when using the LC-MS/MS protocol for the LC-MS/MS analysis, and this is not unexpected. The techniques and protocols are more refined and designed specifically to target

the whole proteome by fragmenting it, followed by deconvolution using computer software to determine the constituent predicted proteins (Zhang *et al*., 2018; Berendsen *et al*., 2017). Certainly, the combination of LC-MS/MS and MALDI-TOF MS has demonstrated exciting possibilities in other bioscience disciplines, for example the characterisation of molecular heterogeneity of tissues in suspected cancer patients (Hoffmann *et al*., 2019). However, the intricacies and complexity involved in sample manipulation prior to analysis negates the use of LC-MS/MS in a routine clinical diagnostic laboratory (Intelicato-Young and Fox, 2013). Improvements such as the screening of targeted metabolites by LC-MS/MS from intact bacterial cells prior to TiO_2-assisted laser desorption/ionisation MS (Zhang *et al*, 2018) are being made in respect of antimicrobial resistance detection in bacteria. Some success has been reported with attempts at serotyping subspecies level identification of *Escherichia coli* and *Salmonella* spp. using LC-MS/MS (Sloan, Wang and Cheng, 2017).

Within the LC-MS/MS dataset, none of the predicted proteins characterised by the data analysis software appears to have a recognised existing role in echinocandin resistance. The majority of predicted proteins appear to be related to ribosomal structure and metabolic pathways, and a large proportion remain uncharacterised following database searches. For example, 31/40 (77.5%) LC-MS/MS predicted proteins present in the NCPF 8814 (echinocandin resistant) extraction B (MALDI-TOF MS specific) were uncharacterised. This is most likely a direct result of the 'bottom up' approach to proteome analysis whereby a large amount of data is generated and published without fully cataloguing and understanding the depths of its content or utility (Boulund *et al*, 2017; Carvalho, Penque and Matthiesen, 2015; Alpi *et al*., 2015; Zhang *et al*., 2013). Much of the data generated by research using approaches to proteomics remain by-products of a singular key factor that any given research project is attempting to find and populates online databases such as UniProt (The UniProt Consortium, 2017; Alpi *et al*., 2015). However, the LC-MS/MS data generated during this study provide potential evidence

for the role of other compensatory mechanisms alongside the genetic mutation of genes in the *FKS* region, which may affect the susceptibility of an organism to the echinocandin class of antifungal agents (Perlin, 2007). For example, the protein Q6FT71 (Table 13) is predicted to have a role in the formation of branched actin networks and actin binding of yeast cells has the potential to directly affect cell integrity, deformability and migration (Mishra, Huang and Balasubramanian, 2014). This may then in turn play a crucial part in the survival of an echinocandin resistant organism, if cytoskeletal structural changes are undertaken as a mechanism of resistance. In fact, actin remodelling has been reported in *C. glabrata* as a direct result of exposure to fluconazole (Bhakt *et al*., 2018) and such an effect may not be limited to a single class of antifungal.

4.3.2 MALDI-TOF MS in the clinical diagnostic mycology laboratory

MALDI-TOF MS produces a spectral profile of ionised biomolecules predominantly consisting of ribosomal and metabolic proteins (Clark *et al*., 2013), and over the past decade manufacturers have packaged the system into platforms that withstand the pressures and restraints of a clinical diagnostic setting (Hou *et al*., 2018; Vella *et al*., 2017; Kothari *et al*., 2014; Vogne *et al*., 2014). Initial costs are high for the acquisition of the platform, but the relative low cost per sample and the ability for offline interpretation outside of machine operation further indicates the utility of MALDI-TOF MS in the clinical laboratory (Tran *et al*., 2015; van Belkum *et al*., 2014).

As stated in Objective 2 of the study aim, the main function of LC-MS/MS analysis to this research was to provide data which could be translated into identified, specific targets for detection by MALDI-TOF MS. However, this study failed to find any correlation between measurable proteins detected by the two methods, even when allowances were made for

detection parameters. This suggests that although the two methods may not be suitable to inform each other (Section 3.2.2 – Section 3.2.4), there may be much to be gained from the concurrent use of both methodologies as a way of developing proteomic knowledge and expanding diagnostic capabilities (Zhu *et al*., 2011). Indeed, some evidence suggests that in other bioscience disciplines the coupling of the two methodologies increases detection sensitivity and mass precision, for example in the rapid detection of urinary biomarkers (Benkali *et al*., 2008).

A key factor to consider when using MS-based proteomic tools in quantitative studies is that neither of the methods presented here is inherently quantitative (Li *et al*., 2017; Geiger *et al*., 2012). Variations in physicochemical properties for peptide species can lead to differences in ionisation efficiencies and variation in signal intensity, potentially creating large windows of mass spectrometric outputs which may differ between methodology, sample and extraction (Fragerquist, 2017; Otto, Becher and Schmidt, 2014). This may provide some explanation for the lack of correlation observed between the two data set outputs, but is also suggests that together, the methods may provide a complete picture of protein profiles present in the extractions (Geiger *et al*., 2012).

It has been documented that the matrix used during MALDI-TOF MS can have a significant impact on the intensity and recovery of mass spectra generated, both in terms of quality and visibility (Yates, Ruse and Nakorchevsky, 2009; Aebersold and Mann, 2003). The use of sinapinic acid (SA), rather than α-cyano-4-hydroxycinnamic acid (HCCA), the standard matrix used for commercial organism identification, can increase the flight of higher molecular weight proteins (Liu and Schey, 2005). Consequently, extracts NCPF 8814 and NCPF 8745 were prepared for MALDI-TOF MS using SA to detect any significant change in spectral data. The use of SA resulted in the production of flat line (zero sum) spectra despite a minimum of three replicated experiments with the same isolates. This is most likely due to a failure in the

optimisation of the system set up for analysis using a different matrix but could also indicate that the change to a different matrix is not suitable for the analysis of fungal proteins. There is evidence to suggest SA has successfully been used to detect single distinctive mass peaks indicating the production of KPC-2 β-lactamase in Enterobacteriaceae (Figueroa-Espinosa *et al*., 2019).

Recent studies have demonstrated the success and consequential clinical utility of MALDI-TOF MS to the early use of appropriate antimicrobial therapy in BSIs predominantly with bacteria (reviewed by Faron, Buchan and Ledeboer, 2017). The growth of resistant and susceptible isolates in the presence of antifungal agents *in vitro,* and then the subsequent analysis by MALDI-TOF MS may increase the success of visualising any proteins where relative abundance is altered during drug exposure, consequently affecting drug susceptibility. Objective 3 of the study aim for this research highlighted the intention to produce protein spectra for echinocandin susceptible and resistant isolates of *C. glabrata* and determine if they could be distinguished using the tools available in the diagnostic mycology laboratory. This was undertaken from several perspectives, including examples of resistant and susceptible isolates alone (Section 3.3.1) and following exposure to antifungal agents (Sections 3.3.2 – 3.3.3). Indeed, several studies have looked at MALDI-TOF MS spectral profiles of organisms exposed to a concentration gradient of antifungal agent. They have suggested that there are observable and statistically significant differences that can be used to determine the antifungal susceptibility or resistance of a given isolate. For example, Saracli *et al*. (2015) demonstrated the power of MALDI-TOF MS to indicate triazole resistance in species of *Candida,* and in particular *C. glabrata*. They passed specific comment on the need to improve the reproducibility of their technique as this differed between species, although they did note a higher level of reproducibility in *C. glabrata* when compared to *C. albicans* and *Candida tropicalis*. To date there is no standardised or commonly accepted methodology available for

the detection of triazole resistance in *Candida* spp.. This possibly indicates that the required manipulation of isolates and data interpretation is counter intuitive to the current methods of detection and susceptibility testing in use in clinical diagnostic mycology laboratories. Whilst there may exist a clinical need to improve rapid reporting of results, the confirmatory work required to standardise and implement such protocols seems to be imbalanced towards maintaining current methodologies (Gitman *et al*., 2017).

However, the initial database interrogation shown in Figure 12 and Table 14 highlights that the spectral dissimilarity demonstrate between isolates in this study is insufficient or too incomplete to prevent cross matching with other database MSPs for *C. glabrata*. This may suggest that differences in spectral patterns could indicate a natural strain variability within the species, rather than an indication of resistance. The given Log (Score) provides a logarithmic scale of compatibility of test spectra and database record whereby 100% match is represented by a score of 3.000. For bacterial organisms, an identification correct to species level requires a score of ≥ 2.300 and to genus level ≥ 2.000 (Clark *et al*., 2013). However, for fungi lower thresholds are accepted, with a score ≥ 2.000 generally being accepted as confirming identity to species level (Fraser *et al*., 2016). Sub-speciation using MALDI-TOF MS has been documented in bacteria (Altman *et al*., 2016; Fagerquist *et al*., 2006), and whilst such methods could be adapted and applied to *C. glabrata*, an investigative research approach of this sort would fall outside of the remit for a clinical laboratory diagnostic test requirement that this study has aimed to find and approaches to sub-speciation were not pursued as part of this project.

More recently, the rapid detection of fluconazole resistance using MALDI-TOF MS has been demonstrated with *C. tropicalis* (Paul *et al*., 2018), an organism for which microbroth dilution susceptibility testing can be difficult (Marcos-Zambrano *et al*., 2016). In addition, Delavy *et al*. (2019) have illustrated the current and possible future benefits in using and adapting

MALDI-TOF MS-based assays to define complete susceptibility patterns in *Candida auris*. Nevertheless, few recent studies have attempted to undertake similar approaches with *C. glabrata* and the echinocandin agents. Early studies by De Carolis *et al*. (2013) and Marinach *et al*. (2009) compared a large dataset of spectral analysis using a composite correlation index (CCI), as first demonstrated by Arnold and Reilly (1998). The data demonstrated the potential to reduce conventional susceptibility testing time from a minimum of 24 hours to 15 hours (De Carolis *et al*., 2013); however, this has not been universally adopted by clinical mycology laboratories. The comparison between resistant and susceptible isolates presented in this study provides further evidence that it is difficult to provide any indication of susceptibility patterns using a proteomic fingerprint in some time scale less than 24 hours (Figure 18), under the experimental conditions employed which are those in routine use in diagnostic mycology laboratories in the UK (Fraser *et al*., 2016; Fatania *et al*., 2015; Gorton *et al*., 2014). One of the reasons that this approach has not yet been implemented as a routine tool in terms of clinical diagnostics may stem from the requirement for each individual isolate to be tested against a single antifungal gradient and, consequently the intense data analysis required by CCI to generate results which severely reduces its utility in real time. This potentially creates a scenario where there is more hands-on analysis for the laboratory technician than the currently accepted microbroth dilution methods of susceptibility testing (CLSI, 2017a; Lockhart *et al*., 2017). Ideally, in order to have true diagnostic value, the detection of echinocandin resistance by MALDI-TOF MS should provide a more efficient, effective and time-reducing methodology than those currently in use. This is predominantly the remit of various methods of susceptibility testing. MALDI-TOF MS detection of resistance would therefore require the ability to detect spectral changes or differences between echinocandin resistant and susceptible isolates at the initial handling and processing stage of the diagnostic investigation. This means without the need for further incubation or additional testing such as growth in the presence of

antifungal agents. This study has served to demonstrate that without such additional handling, MALDI-TOF MS does not provide a viable alternative to currently available methodology for the detection of echinocandin resistance in the clinical diagnostic laboratory.

4.4 The role of research in the clinical mycology laboratory

This study has demonstrated the potential utility of research to routine clinical diagnostics, and the value of research to clinical diagnostics is undeniable. However, there does exist a disparity between the quantity of published data generated by research institutions which suggest potential diagnostic advancement and the application of such data to clinical diagnostics in the laboratory (Ferrante di Ruffano *et al*., 2012a). As reported by Luepke *et al*. (2017) many industrial bioscience companies have shifted away from the development of novel antimicrobial compounds due to scientific, regulatory, and economic obstacles. Often the advancements are made in the areas that are the most financially lucrative or currently on trend, for example the explosion of interest in *C. auris* (Delavy *et al*., 2019; Prakash *et al*., 2016), rather than the areas where small differences, such as the development of technology beyond the purpose for which it was sold, could affect patient impact (Luepke *et al*., 2017; Dittrich *et al*., 2016; Ferrante di Ruffano *et al*., 2012b). There exists a vast amount of resources and funding in the academic study of biological interactions, in particular those of host vs. pathogen (Lum and Cristea, 2017) and pathogen vs. treatment, but it seems very rare that this research directly translates into novel, progressive, or the improvement of clinical diagnostics. It seems logical that research undertaken in the clinical diagnostic laboratory should be focused on improving the patient care experience, and a general shift towards a personalised approach to medicine has been suggested, especially with advances in the understanding of human genomics. However, as reviewed by Maughan (2017), the reality of genomic successes in the

context of cancer treatment and the clinical drive for personalisation are frequently misaligned. It could be argued that all clinical microbiology is personalised, in that the very essence of sampling the microbiota of individual patients automatically demonstrates a personalised approach to treatment and diagnosis.

The use of NGS to further understand and evaluate the total components of the microbial population, or microbiome, of an individual would be considered a move towards a focussed, precision-centred approach to infective management. However, understanding the benefits and advantages to such approaches is only beginning to be considered (Kashyap *et al.*, 2017). There exists an intrinsic disconnect with such studies due to the contrasting experiences of routine diagnostic testing and academic laboratory experimentation, where what works within a developmental laboratory setting, does not automatically translate to a clinical diagnostic setting. Researchers are attempting to facilitate a marriage between the two, but there is little funding, or time provision in general, especially within PHE/NHS laboratories, for laborious and extensive research projects which may or may not benefit the laboratory and improve patient care. Equipment and facilities are at best basic and at worst out-dated, and there exists a constant pressure to present rapid, accurate and informative results which inform clinical decisions with immediate effect thereby reducing the likelihood of successful investigational science in clinical diagnostic laboratories. This highlights the potential benefits and importance of collaborative projects with universities and other academic institutions.

4.5 Critical evaluation of research undertaken as part of this thesis

The main aim of this study was to provide a practical application within the setting of a clinical diagnostic laboratory to address a specific concern. However, there were practical restrictions to the scope of this research. These were based on the equipment available to undertake

investigation, the numbers of clinical isolates available for sampling, and the limitations placed on time for research to be undertaken, as is often the case in routine clinical diagnostic laboratories (McAdam, 2018). A major consequence of this was to provide little scope for investigation of the true nature of proteomic versus genetic markers of resistance alongside phenotypic demonstration of resistance. However, this fell outside the original remit and aim of the work, which had to remain grounded in those tools readily accessible to the clinical diagnostic mycology laboratory. The inability to detect easily identifiable spectral changes in MALDI-TOF MS at incubation times earlier than 24 hours (Figure 18) could be explained by the simple fact that general operational hours of the PHE MRL do not allow for realistic sampling at time points between 4 and 24 hours. The value of providing 24/7 microbiological support to clinical services is well recognised (Özenci and Rossolini, 2019; Dauwalder and Vandenesch, 2014), but as clinical mycology is generally considered a specialist service, this is not currently offered by PHE. The indication that echinocandin resistance in *C. glabrata* remains low in the UK (Fraser *et al*., 2019a), suggests that the empirical use of an echinocandin would still be considered an effective option (Astvad *et al*., 2018; Klingspor *et al*., 2018), and a switch to an oral agent, such as high dose fluconazole, once appropriate testing was completed, would not be time critical in this scenario (Eschenauer *et al*., 2015).

As summarised by Objectives 2 and 3 set out at the beginning of this thesis there was a presumed correlation between genetic mutations and protein alteration either by morphological change, introduction, or relative abundance (Yoo *et al*., 2012; Hoehamer *et al*., 2010; Hooshdaran, Hilliard and Rogers, 2005). The data presented here seem to suggest that such a simplistic approach to the detection of proteomic modification is insufficient to provide the answers required of the objectives. However, the work undertaken in this project has led to the development and re-evaluation of the value that laboratory detection of genetic markers of resistance can provide, alongside standardised methods of resistance classification, and the

potential implications from a clinical perspective in terms of therapeutic guidance (Fraser *et al*., 2019a; McCarty *et al*., 2017).

The detection of the potential protein markers of antifungal resistance, as indicated from LC-MS/MS analysis, by MALDI-TOF MS would require the complete alteration of standardised operational parameters used in the clinical diagnostic microbiology laboratory for MALDI-TOF MS analysis. This is not only in terms of matrix composition and selection, but also mass window detection and sample preparation (Sections 3.2 - 3.3). However, such fundamental changes to the operation of the MALDI-TOF MS in the diagnostic laboratory would dramatically reduce the clinical utility of the suggested targets as rapid identifiers of resistance. The requirement for re-calibration and re-adjustment of equipment has the potential to deconstruct existing workflows and negatively affect the turn-around times in the clinical laboratory, whilst also falling outside the remit of most routine microbiology laboratory MALDI-TOF MS users (Tran *et al.*, 2015; Dingle and Butler-Wu, 2013).

A recently published study from Figueroa-Espinosa *et al*. (2019) has shown success using a double-layered technique with sinapinic acid in the detection of a KPC-2 β-lactamase in carbapenemase producing Enterobacteriaceae with 100% sensitivity. This indicates that if a protein target exists then only minimal modifications may be required for detection. However, the switch between one matrix and another may have ramifications for the total operation of the MALDI-TOF MS in the clinical microbiology laboratory, and so would require a full programme of validation with all microorganisms that are currently identified using this technique.

The initial detection and identification of protein targets of echinocandin resistance in *C. glabrata* was intended to be found using LC-MS/MS, but further exploration of those predicted proteins generated as part of this study to ascertain any role they may have in resistance

mechanisms was not within the remit of this work. Additionally, this would also be beyond the scope of the clinical diagnostic laboratory.

The work presented in this thesis does not attempt to identify any mutations that may be present in *FKS3*, which has recently been shown to act as a negative regulator of echinocandin susceptibility in *C. albicans* (Suwunnakorn *et al*., 2018). Therefore, it is fair to conclude that *FKS3* may also have a role in echinocandin susceptibility in other yeast species. The presence of an overlap in amplification regions may contribute to the poor sequence reads generated for *FKS2*, and the role of *FKS3* in echinocandin susceptibility in *C. glabrata* merits further investigation. Also, no consideration was given for the impact of other cell wall components which may have an effect on the susceptibility profile of an organism, for example chitin, which has been shown to affect susceptibility to CSP (Walker, Gow and Munro, 2013). A multifactorial or cumulative approach to the acquisition of resistance in microorganisms should not be ruled out.

There is a large volume of evidence to support the role of proteomic detection in the identification of antibacterial resistance mechanisms in bacteria (Figueroa-Espinosa *et al.*, 2019; Dortet *et al*., 2018; Zhang *et al*., 2018; Vogne *et al*., 2014), and there is no biological reason why this shouldn't also be the case with fungi. Indeed, proteomic changes directly correlated to antifungal exposure have been demonstrated in *C. albicans* (Hoehamer *et al*., 2010). The challenge for the clinical mycology laboratory is to find a way to approach the investigation of the detection of protein markers of resistance within the confines of the diagnostic setting. Independent studies have demonstrated the ability of MALDI-TOF MS to provide an indication of resistance when considering the azole class of antifungals (Paul *et al*., 2018; Saracli *et al*., 2015; Marinach *et al*., 2009), and this is an area which could be explored further at the PHE MRL. However, it is important to note that there is currently no commercially available or recognised template for the clinical laboratory to detect resistance

using MALDI-TOF MS, and only the continued publication of proof of concept research demonstrates that it should be achievable (Vatanshenassan *et al*., 2018; Paul *et al*., 2018; Vella *et al*., 2017; Saracli *et al*., 2015).

Another limitation of the work undertaken as part of this study is that all growth experiments in the presence of antifungal agent were performed using only CSP to detect evidence of changes in protein profiles. Studies conducted by Bordallo-Cardona *et al*. (2017) and Bizerra *et al*. (2014) have suggested that MCF might be a more effective inducer of *in vitro* resistance. MCF was not used in these experiments due to the difficulty in obtaining a powder or liquid form of the drug to prepare laboratory solutions during the timescale of the research. As a consequence, all MCF testing in this study was performed by E-test only. However, since the project has ended, so too has the licence for MCF, and it is now available from laboratory suppliers.

Previously studied isolates of *C. glabrata* from the NCPF were used in an attempt to identify proteomic signatures suggestive of resistance or susceptibility but, once revived, only two of the five archived strains demonstrated resistance (Table 6), and the reasons for this could be multifactorial as previously discussed (Section 4.2). However, the reliance on the provision of archived examples of echinocandin resistant isolates was critical in the successful completion of Objective 1 and as a consequence all objectives thereafter. This served to highlight the importance of appropriate and accurate curation of culture collections (Forti *et al*., 2016; Becker *et al*., 2015; Lima-Neto *et al*., 2014; Smith, 2012).

A further consequence of a reliance upon archived culture collection isolates, potentially having an impact on the results of this study, was the use of NCPF 8745 as the phenotypical echinocandin susceptible control isolate. This organism was originally added to the NCPF as an example of an echinocandin resistant isolate. Although, as previously discussed, the paperwork to support this accession could not be located so it remains unclear if the revived

isolate's lack of ability to replicate a resistance phenotype reflected changing classification breakpoints (Fraser *et al*., 2019a; CLSI, 2017a; CLSI, 2017b), or whether the *FKS* mutation, which may have been present but not detected at the point of archiving, was lost due to the lack continued echinocandin presence (Imbert *et al*., 2016; Clancy and Nguyen, 2011). Phenotypically susceptible, or wild type isolates from clinical samples are not routinely stored by the NCPF as on-going study is usually dedicated towards those isolates that represented challenges or advancement of knowledge. It may have been possible to select a randomised wild type isolate from a clinical submission during the period of this study however, the use of a curated, tested and already archived isolate such as NCPF 8745 provided a certain element of ease for the immediate continuation of the study.

4.6 Potential future research opportunities arising from the research in this thesis

Further extension of the work presented as part of this thesis should include the expansion of similar projects to other fungal species, especially those currently of national, or indeed international interest such as *C. auris* (Delavy *et al*., 2019; Jeffery-Smith *et al*., 2017). It is crucial for the clinical diagnostic mycology laboratory to continue to provide the platform for development of existing, and introduction of, novel tools to further enhance the clinical and diagnostic interaction so as to maximise patient benefit. The use of MALDI-TOF MS in the determination of antifungal susceptibility patterns has been demonstrated in many pathogenic yeast species including *C. albicans* and *C. glabrata* (Vatanshenassan *et al*., 2018), and the restrictions placed on this study to maintain a direct and immediate clinical utility prevented the full investigation of potential protein markers of resistance in *C. glabrata*.

Specifically, the further exploration into the identity of the proteins represented within the triplet peak signature detected during exposure to echinocandins described in Section 3.3, may provide further information into the interaction of *C. glabrata* and echinocandin antifungal agents *in vitro*. The purification of proteins of known mass, as indicated by MALDI-TOF MS, and their characterisation could be undertaken using 2D-PAGE techniques and protein labelling (Fey *et al*., 1997). This might in turn lead to the discovery of a reproducibly consistent antifungal induced protein change which could be adapted into a diagnostic marker for use in clinical testing (Yoo *et al*., 2012; Yan *et al.*, 2007).

Also, the evidence that supports the use of ANF as a sentinel echinocandin to indicate cross-agent resistance would indicate that further studies, if undertaken, should attempt to utilise ANF and MCF instead of CSP (Pfaller *et al*., 2014c; Espinel-Ingroff *et al*., 2013; Shields *et al*., 2013). As previously mentioned, the role of *FKS3* in echinocandin susceptibility has only recently been described (Suwunnakorn *et al*., 2018), so a more focussed approach on the total role of *FKS* in echinocandin susceptibility could be undertaken, and techniques such as NGS may be beneficial in further elucidating this information.

There is sufficient evidence to suggest that MALDI-TOF MS detection of agents of BSI, including candidaemia, is highly beneficial in terms of laboratory turnaround time and clinical reporting (Angeletti, 2017; Buehler *et al*., 2016). There exists the potential to expand approaches to serological investigations of yeast in blood, predominantly the detection of cell surface antigens such as mannan or (1-3)-β-D-glucan (Mokaddas *et al*., 2011) could be replaced with a MALDI-TOF MS system whereby detection and identification of causative agents is performed directly on blood samples from patients (Curtoni *et al*., 2017; Moore *et al*., 2016; Randazzo *et al*., 2016). This would not only dramatically reduce the notification time of infection, but also the time by which appropriately guided therapy could be administered. This is something that should be considered at the PHE MRL for future diagnostic developments.

It is also worth considering recent shifts towards approaches in microbiology which focus on the total components of the human microbiome, the commensal population of microorganisms that humans host. This perspective of personalised medicine has advanced due to the increased development of high-throughput DNA sequencing technologies such as NGS or WGS, which have enabled the identification of previously non-culturable organisms. They have provided a profile of the total microbiota of a patient (Martin *et al*., 2018), as well as the potential interactions and implications for antimicrobial resistance which can affect and influence human health and disease (Do *et al*., 2018). It has been demonstrated that interactions within the existing microbiological communities of an individual patient can impact the treatment choices available to clinicians (Ferrer *et al*., 2017), the prognoses of some non-infective conditions such as Parkinson's disease (Lubomski *et al*., 2019) or cardiovascular disease (Peng *et al*., 2018) and the outcome of therapeutic decisions (Wong *et al*., 2019; Kashyap *et al*., 2017). Indeed, the interaction between the microbiome and the mycobiome, the fungal component of the commensal microbiota, may have a key role to play in consequences of antimicrobial treatment (Sam, Chang and Chai, 2017). Fungi are generally considered opportunistic pathogens, but when considered as part of a full microbiological profile of an individual it is possible that their presence and consequential impact on treatment selection and resistance profiling may change.

4.7 The future of resistance detection in the clinical mycology laboratory

The rapid detection of existing and emergent resistance before or during treatment with antifungal agents is going to be a vital tool in achieving consistently successful and targeted antifungal therapy. In an era of broad-spectrum antimicrobial resistance, this approach to clinical diagnostics will form the underlying basis of many antifungal stewardship programmes

(Poole, Kidd and Saeed, 2018; Wattal *et al*., 2017). The detection of *FKS* mutations can provide an appropriate indication of the correct antifungal regime selection, and the potential of this study is that it demonstrates how an existing diagnostic laboratory technology can be adapted and primed in the event of rapidly emergent resistance. There are extreme pressures being placed on the use and management of antifungal agents, and this in turn confers an environmental pressure for inducible resistance. By far the largest and most frequently used empirical therapy for the treatment of candidaemia is the azole class of antifungals, and it has been demonstrated that MALDI-TOF MS could have a role in the detection of azole resistance in *C. albicans* (Marinach *et al*., 2009) and some other non-albicans *Candida* sp. (Paul *et al*., 2018; Saracli *et al*., 2015). However, the work presented within this thesis and supported by others, suggests that the physical application of such undertakings does not provide a viable, or significant difference, from the methods that are currently used to detect phenotypic resistance. For example, it has been reported that MALDI-TOF MS analysis provides little to no benefit over standard techniques when used to investigate antifungal susceptibility patterns in *Aspergillus fumigatus*, the most common filamentous fungus to cause infections in humans (Gitman *et al*., 2017). As is the case with some *C. glabrata* isolates and the echinocandin agents, it is possible to see resistance without the presence of known or documented resistance markers (McCarty *et al*., 2018).

It would seem that the adoption of a single system dependent upon the detection of only a genetic or proteomic indicator of resistance in *C. glabrata* would not provide a level of specificity or sensitivity sufficient for complete confidence in the selection of appropriate treatment. Such a methodology is likely to be further confounded by the fastidious growth requirements, and unpredictable nature of handling fungi in the clinical laboratory (Subedi, Jennings and Chen, 2017; Fraczek *et al*., 2014). This suggests that a continued multifactorial approach to resistance detection may be beneficial in the clinical diagnostic mycology

laboratory, particularly in the case of the echinocandin agents and *C. glabrata* as demonstrated by work conducted for this thesis (Fraser *et al*., 2019a). The use of LC-MS/MS and MALDI-TOF MS to identify protein markers of resistance may not be the most appropriate tool in a clinical setting, but if used alongside currently accepted methods of susceptibility testing, they could provide useful additional information to aid clinical decision making.

There is some indication from this thesis that indicates that protein changes alone might not be linked to mutations in the *FKS* gene itself. In fact, a complete proteomic picture may reveal new indicative patterns which in fact represent multiple metabolic processes which could include, but are not limited to, genetic alterations (De Carolis *et al*., 2012; Perlin, 2007). Indeed, the study of proteomics has helped to clarify that it is possible for a large variety of protein species to be derived from a single gene (Jungblut *et al*., 2008; Perlin, 2007).

However, advancements in whole-genome sequencing mean that the focus on approaches to the interrogation of infectious organisms are shifting towards a total genomic approach, where a comprehensive review of all the genetic data of a given clinical isolate is obtained. Advances such as this enable the continual movement of modern medicine closer to individual personalised care (Spettel *et al*., 2019). Although as Balloux *et al*. (2018) have reviewed, significant challenges resulting from the implementation and transference of technologies from academic research to clinical practice need to be overcome to make this a reality for the clinical diagnostic laboratory. This may take the form of complicated methodologies for sample preparation which require condensing to be practicable in the clinical setting, the reduction in financial expenditure required per sample, or the reduction in times required for analysis and return of large datasets generated by WGS platforms, which can often take days to months (Balloux *et al*., 2018; Olaru, *et al*., 2018; Rossen *et al*., 2018).

4.8 Conclusions

Encouragingly, over the three years that isolates were collected for this study, there was only a 0.5% prevalence rate of resistance to echinocandin antifungal agents in *C. glabrata* (Fraser *et al*., 2019a). This provides evidence for a reduction in urgency of the development of rapid detection methods as originally intended and set out as the aim for the work undertaken for this thesis. This also provides some scope for the further development and expansion of understanding how techniques like MALDI-TOF MS can be fully expanded beyond that which they are first adopted. In the case of diagnostic microbiology, rapid organism identification can be utilised to maximum benefit in the clinical diagnostic laboratory by providing more information to guide treatment and therapeutic decisions.

The echinocandin class of antifungals remains an important example of agents which have a unique mode of action within a narrow field of therapeutic options for the treatment of candidaemia. It is imperative that advances and technologies such as demonstrated in this research, are readily deployable should a situation arise where echinocandin resistance increases. This highlights the need for the continued development of new approaches to the detection of resistance, as well as the importance of accurate identification and susceptibility testing, and clinical interpretation, across regional, national and international levels.

Whilst the work undertaken to compile this thesis has been unable to fully complete the four objectives identified to fulfil the aim of the work, it has been successful in changing and further informing clinical reporting in the diagnostic mycology laboratory.

Completion of Objective 1, to create a rapid genomic detection method using pyrosequencing technology (Sections 3.1.2 – 3.1.5) for recognised mutations in *FKS1* and *FKS2* in *C. glabrata*, has been achieved and has been used to demonstrate a full picture of the ongoing prevalence of echinocandin resistance in the UK since testing of echinocandin agents first began in 2003 (Fraser *et al*., 2019a).

Both LC-MS/MS and MALDI-TOF MS have demonstrated the ability to indicate proteins which may play a role in resistance mechanisms in *C. glabrata,* as outlined in Objectives 2 and 3. The limited methodologies in use within the clinical diagnostic laboratory did not allow for the full identification of such proteins and, consequently, their confident adoption as rapid indicators of resistance, without the additional security of susceptibility testing, could not be implemented. Further investigations into the classification and functions of proteins identified in this way may in the future enable the expansion of MALDI-TOF MS as a rapid identifier of resistance.

Data generated by the work undertaken as part of this thesis to complete Objective 4 has demonstrated that a robust database for the detection and differentiation of echinocandin resistant and susceptible isolates of *C. glabrata* by MALDI-TOF MS cannot be created from the small number of *FKS1* and *FKS2* mutants collected during the timescale of this study (Section 3.3.1). However, the usage of MALDI-TOF MS to rapidly identify agents of bloodstream infections, and in particular fungi, has been revolutionary in the diagnostic microbiology laboratory (Faron *et al*., 2017; Clark *et al*., 2013) and continued attempts to increase the scope and utility of this power should be made to further enhance the impact on clinical diagnostic testing.

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6. APPENDICES

Appendix I

Ethics Approval

Public Health England

PHE R&D Office

[redacted]

www.phe.gov.uk

25th June 2015

Mark Fraser
Clinical Scientist
[redacted]

Dear Mark

PROJECT TITLE: Novel approaches to the Detection of Antifungal Resistance in *Candida* spp.

R&D Reference: R&D243

Declaration of Public Health England (PHE) Research Study Approval.

Following an internal review, I write to advise that PHE approve your study 'Novel approaches to the Detection of Antifungal Resistance in *Candida* spp'. This approval is conditional on your study being approved by the Ethics Committee at the University of West England, and a copy of the ethical approval being received in this office.

This approval is effective until project completion. This approval will be terminated if the project should cease to be in the interests of staff safety or you can no longer act as chief investigator and no mutually acceptable replacement can be found. Please ensure that the R&D office is informed of any project amendments.

If you need any further support or information, please do not hesitate to contact the PHE R&D Office, quoting the reference number for your study.

Yours sincerely

[redacted]
Head of Research Governance
Public Health England

University of the West of England
BRISTOL

Mark Fraser
Mycology Reference Laboratory
[redacted]

Faculty of Health & Applied Sciences
[redacted]

UWE REC REF No: HAS.15.08.004

25th August 2015

Dear Mark

Application title: Novel Approaches to the Detection of Antifungal Resistance in Candida spp.

Your ethics application was reviewed by the Faculty Research Ethics Committee and approved as this is a lab based study which does not involve relevant material. The study has been approved subject to adherence to the health and safety protocols outlined in the proposal.

You must notify the committee in advance if you wish to make any significant amendments to the original application using the amendment form at
http://www1.uwe.ac.uk/hls/research/researchethicsandgovernance.aspx

Please note that any information sheets and consent forms should have the UWE logo. Further guidance is available on the web:
http://www1.uwe.ac.uk/aboutus/departmentsandservices/professionalservices/marketingandcommunications/resources.aspx
The following standard conditions also apply to all research given ethical approval by a UWE Research Ethics Committee:

1. You must notify the relevant UWE Research Ethics Committee in advance if you wish to make significant amendments to the original application: these include any changes to the study protocol which have an ethical dimension. Please note that any changes approved by an external research ethics committee must also be communicated to the relevant UWE committee.
2. You must notify the University Research Ethics Committee if you terminate your research before completion;
3. You must notify the University Research Ethics Committee if there are any serious events or developments in the research that have an ethical dimension.

Please note: The UREC is required to monitor and audit the ethical conduct of research involving human participants, data and tissue conducted by academic staff, students and researchers. Your project may be selected for audit from the research projects submitted to and approved by the UREC and its committees.
We wish you well with your research.
Yours sincerely

[redacted]
Chair
Faculty Research Ethics Committee

c.c [redacted]

Appendix II

Pyrosequencing *FKS* FASTA format file for *C. glabrata*

This is an essential component for the rapid identification of sequencing results. The Identifire® software requires a database to search the sequences against. This document was used to create that database. Hot spot mutations/alterations highlighted in RED. Mutations that confer phenotypic resistance are indicated by the amino acid (Aa) substitution and gene position (XXX) in the format: AaXXXAa. WT = wild type variant.

>CGFKS1_WT.1
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>CGFKS1_WT.2
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>CGFKS1_WT.3
AAAATTCTGATTGGATCTCTTAGAGATAGAATCAAGAAGTAGTA

>CGFKS1_WT.4
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>CGFKS1_WT.5
AAAATTCTGATTGGATCTCTTAGTGATAGAATCAAGAAGTAGTA

>CGFKS1_WT.6
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>CGFKS1_WT.7
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>CGFKS1_WT.8
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>CGFKS1_WT.9
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>CGFKS1_WT.10
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>CGFKS1_F625S.TCT
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>CGFKS1_F625S.TCC
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>CGFKS1_F625S.TCA

AAAATTCTGATTGGATCTCTTAGAGATAGAATCAATGAGTAGTA

>CGFKS1_F625S.TCG
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>CGFKS1_F625S.AGT
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>CGFKS1_F625S.AGC
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>CGFKS1_S629.CCC
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>CGFKS1_S629P.CCA
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>CGFKS1_S629P.CCG
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>CGFKS1_D632E.GAA
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>CGFKS1_D632E.GAG
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>CGKFS1_D632G.GGT
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>CGFKS1_D632G.GGC
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>CGFKS1_D632G.GGA
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>CGFKS1_D632G.GGG
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>CGFKS1_D632Y.TAT
AAAATTCTGATTGGCTCTCTTAGAGATAGAATCAAGAAGTAGTA

>CGFKS1_D632Y.TAC
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>CGFKS2_WT.2
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>CGFKS2_WT.20
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>CGFKS2_WT.21
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>CGFKS2_deletionF658
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAGAAGTA

>CGFKS2_deletionF659
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAGAAGTA

>CGFKS2_F659S.TCT
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAAGAGAAGTA

>CGFKS2_F659S.TCC
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAGGAGAAGTA

>CGFKS2_F659S.TCA
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAATGAGAAGTA

>CGFKS2_F659S.TCG
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAACGAGAAGTA

>CGFKS2_F659S.AGT
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>CGFKS2_F659S.AGC
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>CGFKS2_F659V.GTT
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAAACGAAGTA

>CGFKS2_F659V.GTC
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAGACGAAGTA

>CGFKS2_F659V.GTA
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAATACGAAGTA

>CGFKS2_F659V.GTG
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAACACGAAGTA

>CGFKS2_F659Y.TAT
AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAATAGAAGTA

>CGFKS2_F659Y.TAC

AAAATTCTGATAGGGTCTCTTAGAGACAAAATCAAGTAGAAGTA

>CGFKS2_L662W.TGG
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>CGFKS2_S663P.CCT
AAAATTCTGATAGGGTCTCTTAGAGGCAAAATCAAGAAGAAGTA

>CGFKS2_S663P.CCC
AAAATTCTGATAGGGTCTCTTAGGGGCAAAATCAAGAAGAAGTA

>CGFKS_S663P.CCA
AAAATTCTGATAGGGTCTCTTAGTGGCAAAATCAAGAAGAAGTA

>CGFKS2_S663P.CCG
AAAATTCTGATAGGGTCTCTTAGCGGCAAAATCAAGAAGAAGTA

>CGFKS2_S663F.TTT
AAAATTCTGATAGGGTCTCTTAGAAACAAAATCAAGAAGAAGTA

>CGFKS2_S663F.TTC
AAAATTCTGATAGGGTCTCTTAGGAACAAAATCAAGAAGAAGTA

>CGFKS2_S663Y.TAT
AAAATTCTGATAGGGTCTCTTAGATACAAAATCAAGAAGAAGTA

>CGFKS2_S663Y.TAC
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>CGFKS2_L664R.CGT
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>CGFKS2_L664R.CGC
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>CGFKS2_L664R.CGA
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>CGFKS2_L664R.CGG
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>CGFKS2_L664R.AGA
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>CGFKS2_L664R.AGG
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>CGFKS2_P667T.ACT
AAAATTCTGATAGTGTCTCTTAGGTACAAAATCAAGAAGAAGTA

>CGFKS2_P667T.ACC
AAAATTCTGATGGTGTCTCTTAGGTACAAAATCAAGAAGAAGTA

>CGFKS2_P667T.ACA
AAAATTCTGATTGTGTCTCTTAGGTACAAAATCAAGAAGAAGTA

>CGFKS2_P667T.ACG
AAAATTCTGATCGTGTCTCTTAGGTACAAAATCAAGAAGAAGTA

Appendix III

Preparation of samples for LC-MS/MS at the Proteomics facility, University of Bristol

Extract A samples were re-suspended in 100µL of 8M urea plus protease inhibitors, 1% nonylphenyl-polyethylene glycol (NP-40), 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS) and then made up to 185 µL with 100 mM triethylammonium bicarbonate (TEAB). Next, 10 µL of 200 mM tris-(2-carboxyethyl)-phosphine (TCEP) was added and the samples were incubated at 55°C for 1 hour. Following this, 10 µL of 375 mM iodoacetamide was added and the samples incubated in the dark at room temperature for 30 minutes. After this time, 6 volumes of ice-cold acetone were then added to each tube and the samples were allowed to precipitate at -20°C overnight. The samples were next centrifuged at 8,000g for 10 minutes at 4°C, the supernatant was removed, and the pellets were allowed to air dry for 3 minutes before being re-suspended in 50 µL 50 mM TEAB. Finally, 2.5 µg sequencing-grade trypsin was added to each tube and the samples were incubated at 37°C overnight. The resulting peptides were desalted using SepPak cartridges (Waters, Milford, Massachusetts, USA) according to the manufacturer's instructions and the eluate was evaporated to dryness and re-suspended in 1% formic acid prior to LC-MS/MS analysis using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific).

LC-MS/MS Analysis

Peptides in 1% (vol/vol) formic acid were injected onto a 250 mm x 75 µm Acclaim PepMap C18 nano-trap reverse phase analytical column (Thermo Scientific), after washing with 0.5% (vol/vol) acetonitrile, over a 150-minute organic gradient. The organic gradient consisted of 7 gradient segments: 1-6% solvent B over 1 minute, 6-15% solvent B over 58 minutes, 15-32% solvent B over 58 minutes, 32-40% solvent B over 5 minutes, 40-90% solvent B over 1 minute,

held at 90% solvent B for 6 minutes and then reduced to 1% solvent B over 1 minute with a flow rate of 300 nL/min. Solvent A was 0.1% formic acid and solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionised by nano-electrospray ionisation at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250°C.

Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300-2000 and the top ten multiply charged ions in each duty cycle were selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count,1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were: normalised collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

Appendix IV

Table 1. Subculture and resulting macro-colonies of NCPF isolates exhibiting changing patterns of susceptibility *in vitro* to anidulafungin. Table 9 (page 57) shows selected examples from this complete data set. This table shows the results from the subculture and macro-colony analysis generated from the passage of echinocandin susceptible NCPF isolates of *C. glabrata* in the continued presence of anidulafungin. The MIC of each collected isolate is provided.

Key: Number = testing identifier Strain = NCPF isolates, or macro colony identifier e.g. NCPF subculture-macro, Sub = number of subculture, macro = number of macro colony, MIC = minimum inhibitory concentration of anidulafungin at 24 hours (µg/mL) or caspofungin in denoted by C, *FKS1/2* = Identifire® sequence identification, Mutation = Mutation = No (N) or Yes (Y) and if representing a hot spot then with the result: *FKS* gene (1/2), amino acid (Aa) substitution and gene position (XXX) in the format: 1/2 – AaXXXAa, or Undetermined (?), Score = Percentage of match between sequence read and *FKS* sequence database.

#	Strain	Sub	Macro	MIC (µg/mL)	FKS1	FKS2	Mutation	Score
2	8714	0	0	0.064 C				
10	8714	1	0	0.012	CGFKS1_WT.3	CGFKS2_WT.1		100/92.9
7	8714	2	0	0.008	CGFKS1_WT.3	CGFKS2_L662W.TGG	?	100/91.9
14	8714	3	0	0.006	CGFKS1_WT.3	CGFKS2_L662W.TGG	?	100/95.6
20	8714	4	0	0.006	CGFKS1_WT.3	CGFKS2_WT.1		100/92.9
29	8714	5	0	0.006	CGFKS1_WT.3	CGFKS2_WT.1		100
45	8714	6	0	0.006	CGFKS1_WT.3	CGFKS2_WT.1		100
	8714	7	0	0.008	CGFKS1_WT.3	CGFKS2_WT.1		100
3	8715	0	0	0.064 C	CGFKS1_WT.3	CGFKS2_WT.8		100/86.9
9	8715	1	0	0.016	CGFKS1_WT.3	UNDETERMINED		100
11	8715	2	0	0.012	CGFKS1_WT.3	UNDETERMINED		100
13	8715	3	0	0.008	CGFKS1_WT.3	CGFKS2_WT.8		100/87
19	8715	4	0	0.003	CGFKS1_WT.3	UNDETERMINED		100
28	8715	5	0	0.008	CGFKS1_WT.3	CGFKS2_WT.8		100/78
44	8715	6	0	0.012	CGFKS1_WT.3	CGFKS2_WT.8		100/98
	8715	7	0	0.012	CGFKS1_WT.3	CGFKS2_WT.8		100/85
17	8715	2	1	0.5	CGFKS1_WT.3	ΔF659	?	100/86.4
	8715 2-1	1	0	1	CGFKS1_WT.3	CGFKS2_WT.8		100/87
30	8715 2-1	2	1	0.25	CGFKS1_WT.3	CGFKS2_WT.8		100/97.2
21	8715 2-1	2	2	2	CGFKS1_WT.3	CGFKS2_WT.8		100/97
24	8715 2-1	2	3	3	CGFKS1_WT.3	UNDETERMINED		100
25	8715 2-1	2	4	2	CGFKS1_WT.3	CGFKS2_WT.8		100/95
22	8715 2-1	2	5	2	CGFKS1_WT.3	UNDETERMINED		100
16	8715 2-1	2	6	2	CGFKS1_WT.3	CGFKS2_WT.8		100/70
23	8715 2-1	2	7	4	CGFKS1_WT.2	UNDETERMINED		88.7
32	8715 2-2-1	1	0	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.8		100/98

35	8715 2-2-2	1	0	0.5 (0.38)	CGFKS1_WT.3	CGFKS2_WT.8		100/87
31	8715 2-2-3	1	0	2	CGFKS1_WT.3	CGFKS2_WT.8		100/87
26	8715 2-2-4	1	0	0.5 (0.38)	CGFKS1_WT.3	CGFKS2_WT.8		100/87
36	8715 2-2-5	1	0	1 (0.75)	CGFKS1_WT.3	CGFKS2_WT.8		100/67
34	8715 2-2-6	1	0	1	CGFKS1_WT.9	CGFKS2_WT.8		89/91.2
33	8715 2-2-7	1	0	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.8		1000/92
42	8715 2-4	1	1	2	CGFKS1_WT.3	CGFKS2_WT.8		100/79
40	8715 2-4	1	2	1	CGFKS1_WT.3	CGFKS2_WT.8		100/86
51	8715 2-4-1	2	1	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.8		100/88
60	8715 2-4-1	2	2	2	CGFKS1_WT.3	CGFKS2_WT.8		100/91.4
57	8715 2-4-2	1	1	0.75C, 0.38 A	CGFKS1_WT.3	CGFKS2_WT.8		100/67
56	8715 2-4-2	1	2	1.5C, 1A	CGFKS1_WT.3	CGFKS2_WT.8		100/75
41	8715 2-6	1	1	2	CGFKS1_WT.3	CGFKS2_WT.8		100/82
37	8715 2-6	1	2	2 (1.5)	CGFKS1_WT.9	CGFKS2_WT.8		100/83
39	8715 2-6	1	3	1	CGFKS1_WT.3	CGFKS2_WT.8		85/87
38	8715 2-6	1	4	1	CGFKS1_WT.3	CGFKS2_WT.1		100
59	8715 2-6-1	2	1	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.1		100
58	8715 2-6-1	2	2	2	CGFKS1_WT.3	CGFKS2_WT.1		100/98
46	8715 2-2-1	2	0	0.5	CGFKS1_WT.3	CGFKS2_WT.1		100
49	8715 2-2-2	2	0	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.1		100/97
53	8715 2-2-3	2	0	0.5 (0.38)	CGFKS1_WT.3	CGFKS2_WT.1		100
50	8715 2-2-4	2	0	2	CGFKS1_WT.3	CGFKS2_WT.1		100/89
52	8715 2-2-5	2	0	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.1		100/67
48	8715 2-2-6	2	0	2 (1.5)	CGFKS1_WT.3	CGFKS2_WT.1		100/87
47	8715 2-2-7	2	0	1	CGFKS1_WT.3	CGFKS2_WT.1		100
1	8745	0	0	0.016 C	CGFKS1_WT.3	CGFKS2_WT.1		100/87
8	8745	1	0	0.016	CGFKS1_WT.3	CGFKS2_WT.1		100/96.6
15	8745	2	0	0.008	UNDETERMINED	CGFKS2_WT.3		/92.9
12	8745	3	0	0.008	CGFKS1_F625S	CGFKS2_WT.3	?	81.6/93.4

18	8745	4	0	0.25C, 0.047A	CGFKS1_WT.3	CGFKS2_WT.1		100/89.2
27	8745	5	0	0.064	CGFKS1_WT.3	CGFKS2_WT.1		100
43	8745	6	0	0.032	CGFKS1_WT.3	CGFKS2_WT.1		100