Resistance to echinocandin antifungal agents in the United Kingdom in clinical isolates of *Candida glabrata*: Fifteen years of interpretation and assessment

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ABSTRACT

Candidaemia is widely reported as the fourth most common form of bloodstream infection worldwide. Reports of breakthrough cases of candidaemia are increasing, especially in the context of a move away from azole antifungals as prophylactic or first line treatment towards the use of echinocandin agents. The global evaluation of echinocandin antifungal susceptibility since 2003 has included switches in testing methodologies and the move to a sentinel echinocandin approach for classification reporting. This study compiles previously unpublished data from echinocandin susceptibility testing of UK clinical isolates of *C. glabrata* received at the Public Health England Mycology Reference Laboratory from 2003 to 2016, and re-evaluates the prevalence of resistance in light of currently accepted testing protocols. From 2015 onwards, *FKS* gene mutation detection using a novel Pyrosequencing® assay was assessed as a predictor of echinocandin resistance alongside conventional susceptibility testing.

Overall, our data show that echinocandin resistance in UK isolates of *C. glabrata* is a rare phenomenon and prevalence has not appreciably increased in the last 14 years. The pyrosequencing assay was able to successfully detect hot spot mutations in *FKS1* and *FKS2*, although not all isolates that exhibited phenotypic resistance demonstrated detectable hot spot mutations. We propose that a rapid genomic based detection method for *FKS* mutations, as part of a multifactorial approach to susceptibility testing, could help provide accurate and timely management decisions especially in regions where echinocandin resistance has been reported to be emerging in this important pathogen.

(Word Count: 239)

INTRODUCTION

Candidaemia, which is widely reported as the fourth most common form of bloodstream infection worldwide, presents a considerable challenge to modern medicine. An increase in resistance to established antifungal agents and changing patient demographics are helping to widen the spectrum of species able to cause infection [1-3]. The appropriate use of antifungal drugs is essential for timely and successful clinical outcomes, helping to reduce the burden of emergent resistance and financial strain upon healthcare providers [4-6]. In most countries, *Candida glabrata* continues to be the second most commonly isolated cause of candidaemia after *Candida albicans*, with some healthcare providers indicating an increase in prevalence [7]. A member of the Nakaseomyces clade alongside *C. nivariensis* and *C. bracarensis*, *C. glabrata* presents added complications for clinicians as a significant proportion of isolates exhibit elevated minimum inhibitory concentrations (MICs) to the triazole antifungal agents in general *in vitro*, and in particular to fluconazole [8-10].

The echinocandin antifungal class primarily consists of caspofungin (CSP), anidulafungin (ANF) and micafungin (MCF). These agents are acylated cyclic hexapeptides, which demonstrate some fungicidal activity by non-competitively inhibiting β-1, 3-glucan synthase and represent a niche class of antifungal agents for treatment of candidaemia. However, breakthrough cases of infection are increasingly being reported, especially in the context of an increase in echinocandin use for prophylactic coverage or as a first line treatment alternative to fluconazole [11-16]. Indeed, several studies have reported the

potential for rapid acquisition of echinocandin resistance [11-12], with resistance rates as high as 13.5% observed within one US healthcare centre[13].

Well-established methodologies for *in vitro* susceptibility testing in the clinical laboratory are used to determine the MIC of an antifungal agent [17-18]. However, methods of testing are both labour intensive and time consuming with results typically available to clinicians only 48-72 hours post isolation [17]. This can result in poor or ineffective empirical treatment decisions that require alteration following interpretation of laboratory results [18]. Further compounding such method restrictions is the inter-laboratory variability demonstrated when CSP alone is used for determination of MIC value. Consequently it has been reported that the use of CSP could lead to an over reporting of resistance [19]. Efforts to streamline established methods have demonstrated some success; especially with *C. glabrata* although the time from isolation to result is still limited by the requirement for further or repeat growth of the organism before an interpretation of results can be made, for example between 24-48 hours for E-test [17-20].

A major mechanism for echinocandin resistance in *Candida* sp. has been attributed to so-called hot spot mutations within the *FKS* gene. First described in *Candida albicans*, this gene has been shown to encode a large integral membrane protein suspected to be β-1, 3-glucan synthase [21]. Distinct mutations within this gene region have been described in isolates demonstrating resistance to echinocandin antifungal agents [22-24], and it has been suggested that the presence

of an *FKS* mutation rather than an MIC value is an independent risk factor in the prediction of the outcome of echinocandin usage [24-25].

In the last decade, genomic and proteomic technologies such as Pyrosequencing® and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) have revolutionised the speed and accuracy of identification of clinical isolates of fungi in the diagnostic laboratory [26-28]. While work continues to evaluate the use of proteomics for susceptibility testing [30-31], there remains a clinical need for accurate and rapid detection of resistance. Although genomic approaches such as Pyrosequencing for isolate identification have somewhat been eclipsed by MALDI-TOF MS, the repurposing of this technology due to its unique chemistry, speed and ease of use for reliable sequencing of short regions of DNA could be useful for the detection of resistance markers. Other alternative rapid PCR based methodologies and asymmetric PCR coupled with allele-specific molecular beacons have also previously been employed to determine the presence of *FKS1* and/or *FKS2* mutants [31-32].

The UK National Mycology Reference Laboratory (MRL) at Public Health England (PHE) provides clinical and diagnostic services for the entire United Kingdom and processes in excess of 5000 isolates of pathogenic yeast for susceptibility testing annually. Isolates are referred from local, regional and national centres throughout the UK and Ireland. The main aim of this study was to retrospectively analyse MIC data generated at the MRL from the start of echinocandin testing in 2003 up to the end of 2016. This data has been reviewed with consideration to the patterns of changing opinion regarding methodological restrictions, shifting

clinical breakpoints and inter-laboratory variations to provide a clearer picture of the prevalence of echinocandin resistance in the UK. From 2015 onwards, a Pyrosequencing platform was employed in parallel as a real-time detector of *FKS* mutations, and therefore echinocandin resistance.

MATERIALS & METHODS

Clinical Isolates for Minimum Inhibitory Concentration (MIC) testing

All isolates were subcultured onto Sabouraud dextrose agar with chloramphenicol (SABC; PO0161A; Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 30°C for 48 hours.

Minimum inhibitory concentrations (MIC) were collated for all clinical isolates of *C. glabrata* referred to the PHE MRL between 2003 and July 2016. Isolates were identified according to standard protocols employed at our laboratory as follows. Isolates received between 2002 and December 2007 were identified by a combination of AUXACOLOR2/API 20C in conjunction with 26S rRNA gene sequencing [33]; from January 2008 through May 2012 all isolates were identified by Pyrosequencing of a portion of the internal transcribed spacer region 2 [28]; from May 2012 through July 2018 all isolates were identified by MALDI-TOF [27].

Susceptibility testing was either performed using E-test (MCF from 2002; CSP from 2012; ANF as confirmation of resistance) as provided by BioMérieux (Product code 532418) and using RPMI agar (BioMérieux AEB122180) or by CLSI microbroth dilution (CSP between 2002 and 2012; ANF from 2002) as previously described [17-18]. Throughout the time period, various breakpoints were used in accordance with published data at the time [34-38]. At the PHE MRL, susceptibility cut off values of 0.25µg/mL for CSP and ANF, and 0.125µg/mL for MCF were used to indicate the likelihood of resistance. This is primarily because an interpretation of "intermediate" is of little value to the clinician, and would ultimately result in that particular agent not being used but also as this value has

151 previously demonstrated high sensitivity and specificity for the selection of FKS
152 mutants [39].
153 Archived isolates of *C. glabrata* stored in the National Collection of Pathogenic
154 Fungi (NCPF) were used as control strains and were selected based upon
155 previous antifungal resistance testing, where phenotypic resistance to the
156 echinocandin class of antifungal agents had been demonstrated; generally this
157 was achieved by CLSI microbroth dilution testing against CSP [36]. All archived
158 isolates were subjected to confirmatory echinocandin resistance testing by
159 established methods prior to sequencing analysis for *FKS* mutations [37].
160
161 Pyrosequencing and rapid detection of FKS mutants
162 During the period from July 2015 to July 2018, all clinical isolates of *C. glabrata*
163 submitted to the PHE MRL for susceptibility testing which exhibited phenotypic
164 resistance to an echinocandin agent were subjected to Pyrosequencing for the
165 detection of *FKS* mutations.
166 Biotinylated forward primers were created based on the position of *FKS*
167 mutations that confer echinocandin resistance to strains *in vitro* as previously
168 described for *Saccharomyces cerevisiae* and *Candida albicans* [40-41] and resembled
169 Cg*FKS1*, GenBank accession no. KF211456.1 [12] and Cg*FKS2*, GenBank accession
170 no. HM366442.1[42]. The reverse primer (20µM CgFKS1/2 reverse) was used as
171 the Pyrosequencing analysis primer. PyroMark™ID (Biotage AB, Uppsala,
172 Sweden) was used to perform the Pyrosequencing protocol as previously
173 described [28]. To enable the real-time analysis of sequences generated by the
174 IdentiFire® Pyrosequencing analysis software (Biotage AB, Uppsala, Sweden),

175 the creation of a FASTA format file was necessary for each possible mutation and
176 wild type (WT) sequence within *FKS1* and *FKS2*.
177
178 RESULTS & DISCUSSION
179
180 Re-evaluation of MIC data in the context of changing laboratory protocols
181
182 In total from 2003 to 2018, 7,225 clinical isolates of *C. glabrata* were tested for
183 echinocandin susceptibility at the PHE MRL. Between 2003 and 2016 there was a
184 considerable shift in MIC values reported for *C. glabrata* when tested against CSP
185 (Table 1). This followed the acknowledgement that species-specific breakpoints
186 were required [37] and as reported by Espinel-Ingroff *et al*. (2013), that CLSI
187 testing of CSP was considered too variable between laboratories to reliably
188 indicate resistance. In the first decade of echinocandin testing from 2003 to
189 2013, MIC values by CLSI methodology indicated resistance prevalence ranging
190 from 0.3- 7.9% with a mean of 3.4% using the CLSI breakpoints in operation at
191 the time of antifungal susceptibility testing. In fact, when current breakpoints are
192 retrospectively applied to this historical data, the vast majority of isolates
193 exhibited MICs which fall in the non-susceptible range, in complete agreement
194 with Espinell-Ingroff *et al*. that caspofungin susceptibility testing by broth
195 microdilution artificially inflates resistance rates[19]. With the introduction of the
196 use of ANF as the sentinel echinocandin and E-test for specific CSP testing, this
197 prevalence range was reduced to 0.9-2.7% with a mean of 1.5%. Data from 2012
198 is difficult to interpret due to the fact that both testing methods were in use and
199 the breakpoints were under review so the point prevalence rate of 5.9% is

200 artificially raised and does not indicate emergent or outbreak resistance. In fact,
201 if the data is re-assessed using the 2017 higher breakpoint cut off then
202 prevalence drops to 1.1% (2/185) [37].
203 The total prevalence of 2.8% resistance to micafungin is artificially elevated in
204 this data set as it was principally employed to confirm resistance detected with
205 the other echinocandins prior to 2015.
206
207 This study provides evidence that the prevalence of echinocandin resistance in
208 clinical isolates of *C. glabrata* in the UK remains very low at 0.55% between 2015
209 and 2018 when testing is optimised. This correlates with studies conducted in
210 other European countries and China [10, 43-45], and appears consistent with the re-
211 evaluated *in vitro* data generated prior to this timeframe.
212
213 Analysis of FKS mutation detection as a predictor of resistance
214
215 Of the 2713 *C. glabrata* isolates subjected to antifungal susceptibility testing at
216 the PHE MRL between July 2015 and July 2018, fifteen (0.55%) exhibited some
217 degree of phenotypic resistance to one or more echinocandin agent. The details
218 for each isolate can be found in Table 2. Eleven isolates demonstrated MICs in
219 the resistant range for at least two of the echinocandin antifungal agents
220 (isolates 61, 71-76, 78-81), two further isolates had resistant MICs with
221 caspofungin, intermediate MICs with anidulafungin but susceptible MICs with
222 micafungin (isolates 67 and 77), and the final 2 isolates (68 and 70) had
223 intermediate MICs with caspofungin alone, which was the only echinocandin
224 tested. Of particular note, 6/15 (40%) isolates originated from blood, 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 months and 6 months apart.

FKS mutations detected by Pyrosequencing are displayed in Table 3. In total, 6/15 (40%) 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). For *FKS2*, 4/15 (26.7%) isolates showed an indisputable mutation, with again 4/4 (100%) representing a serine to proline substitution but at position 663 (S663P). The presence of a single mutation in either *FKS1* or *FKS2* was sufficient to confer resistance to all echinocandin agents. Isolates 71 and 72 may have mutations in both regions but despite repeat testing, the sequence homology score for *FKS2* was never greater than 90% as shown in Table 3. Isolates 61 and 80 failed to show any evidence of amplification for the *FKS2* region on repeated attempts and are listed as inconclusive for mutations in this region. In the case of isolate 80, *FKS1* demonstrated a wild type sequence. Of those isolates demonstrating some degree of phenotypic resistance, 4/15 (26.7%) did not apparently possess a mutation in either *FKS1* or *FKS2* (isolates 67, 68, 70 and 77). However, all 4 isolates demonstrated MIC values that flanked the cut-off value (0.25 – 0.5μg/mL) and as such could be considered phenotypically borderline resistant/non-susceptible. Generally, when the MIC was at least 2 doubling dilutions greater than the susceptibility cut-off value, there was an associated *FKS* mutation in either *FKS1* or *FKS2*. The exception was

249 isolate 71 where MICs to ANF and CSP were within 2 doubling dilutions from the
250 cut off value but an *FKS1* mutation was detected.
251
252 Developing technological approaches using MALDI-TOF MS have demonstrated
253 their potential for the detection of resistance mechanisms. However, the financial
254 constraints of acquiring the platforms, coupled with time limitations, whereby a
255 minimum incubation time of 6 hours post isolation is required prior to a result
256 being reported [29-30], suggests there is scope for alternative methodologies. The
257 Pyrosequencing assay described here has a minimum detection time of 4 hours
258 post isolation, and many clinical laboratories may already have access to the
259 platform without further expenditure. Whilst this study used log phase growth,
260 freshly subcultured from referred isolates, it would be possible to perform the
261 Pyrosequencing assay directly on isolates at the point of receipt or isolation as
262 previously demonstrated [27-28]. Interestingly, some studies have evaluated the use
263 of Pyrosequencing directly from blood cultures and found >90% success rates in
264 identifying bacterial species [46-47]. This suggests there exists the possibility of
265 running the *FKS* assay without the need for secondary subculture or incubation
266 of tests and thereby reducing the time to implement corrective therapeutic
267 management.
268 This highlights the potential for the use of Pyrosequencing as a baseline
269 screening procedure or empiric antifungal triage service for all isolates referred
270 to the reference laboratory, or in centres were emergent resistance has been
271 demonstrated. This could significantly reduce time and financial impacts, with a
272 possible reduction in the selection/alteration of antifungal regimes within 24
273 hours, a direct contrast to many susceptibility test protocols [17-18].

In agreement with the published literature, the current study has underscored the utility of *FKS* mutation detection in predicting frank echinocandin resistance, in that all isolates exhibiting resistant MICs with at least two echinocandin antifungal agents had demonstrable *FKS* hot spot mutations. However, three isolates that did not have *FKS* mutations phenotypically, demonstrated MICs that would be considered intermediate to CSP and as such they would not be recommended as therapeutic options. A similar situation was reported recently from a US study [48] that found phenotypic non-susceptibility to micafungin in the absence of detectable *FKS* mutations. This further supports the continued role of susceptibility testing of individual isolates in the determination of appropriate antifungal agents for therapeutic selection as advised in the current guidelines of the ESCMID fungal infections study group, as well as the continued use of anidulafungin or micafungin as indicators of true echinocandin resistance [38-39]. It was difficult to ascertain a high confidence sequence read (>99% coverage/identity) for *FKS2* with two of the clinical isolates (isolates 71 and 72). This could be due to multiple factors, including short primer length, or quality of the initial PCR amplification products. The two isolates are unrelated in terms of isolation and geographical region, and the suggested mutation (S663F), if present, has previously been associated with echinocandin resistance [22]. However, since both isolates also harboured S629P mutations in *FKS1*, we cannot conclude definitively on the presence of the purported *FKS2* mutation on the basis of observed phenotypic echinocandin resistance. It has previously been demonstrated that the efficiency of Pyrosequencing is optimised over a region of 30-35 bases [28]. In order to capture all of the possible *FKS* hot spot mutation

299 regions, this had to be extended to up to 45 bases and it is possible that the
300 internal chemistry of the assay becomes too unreliable at this length, resulting in
301 low score matches as observed with some *FKS2* sequences. Sequence homology
302 scores less than 100% must be treated with caution when a single base point
303 alteration can result in the mutation of the gene, especially if it occurs within a
304 hot spot region. This study does not account for mutations that may be present
305 in *FKS3* recently shown to act as a negative regulator of echinocandin
306 susceptibility in *C. albicans* [49].
307
308 It is important to note that as a reference laboratory, PHE MRL data is often
309 skewed due to the fact that we receive predominantly those isolates that regional
310 or local laboratories find difficult to interpret or require confirmatory testing for.
311 Even so, the MIC data collected from testing using E-test for CSP and MCF and
312 CLSI for ANF prior to the *FKS* mutation detection trial, demonstrates a low
313 echinocandin resistance rate of around 0.9-1.5%, similar to that reported
314 recently from a US study which found reduced micafungin susceptibility and/or
315 demonstrable FKS mutations in 33 of 3876 (0.85%) isolates of *Candida* spp and
316 12/832 (1.44%) of isolates of *C. glabrata* [48]. However, with the increase in
317 commercially available microbroth dilution systems which include all three
318 currently available echinocandin agents (e.g. Sensititre Yeast One™, Thermo
319 Fisher), and as more laboratories move towards in-house testing it is important
320 that laboratories using these platforms are aware of the difficulties in
321 interpreting CSP MIC values and potential consequences. When testing CSP,
322 results may be reported as intermediate or resistant however CLSI recommend
323 that confirmatory testing is performed either using ANF or MCF, DNA analysis to

confirm *FKS* hot spot mutation or by sending to a reference laboratory [38]. Regardless of CSP MIC result, CLSI recommend that if either of these first two criteria is fulfilled then pan-echinocandin resistance is confirmed and should be reported.

Interestingly, 3/5 (60%) of the revived NCPF isolates did not exhibit any phenotypic resistance to the echinocandin agents, and indeed did not possess demonstrable mutations in *FKS1* or *FKS2* (data not shown). This finding may indicate the potential loss of acquired resistance mechanisms post storage or revival, mis-cataloguing of accession cultures, or more likely is due to re-interpretation of MIC results in light of changing susceptibility breakpoints. This serves to highlight the importance of validating culture collection strains or strains stored for long periods of time prior to use in research [50].

In conclusion, the rapid detection of existing, or emergent resistance before or during treatment with antifungal agents is going to be a vital tool in allowing successful targeted antimicrobial therapy. This will form the underlying basis of many antifungal stewardship program's as we enter an era of broad spectrum antimicrobial resistance. The detection of *FKS* mutations can provide an appropriate indication of the correct antifungal regime selection and the power of this study is that it demonstrates how an existing diagnostic laboratory technology can be adapted and primed for the eventual emergence of resistance. With very little funding or motivation for the development of novel antifungal agents, the echinocandin class of antifungals remains an important example of unique mode of action agents within a narrow field of therapeutic options for the

treatment of candidaemia. It is imperative that advances and technologies such as demonstrated in this study, are readily deployable should a situation arise where echinocandin resistance increases. This highlights the need for the development of resistance detection approaches and the importance of accurate susceptibility testing and interpretation at local and regional levels.

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Disclosure of Conflict of Interest

The authors have no conflicts of interest to declare

Authorship declaration

MF and AMB designed the study
MF performed the experiments
MF, AMB, LL and RT analysed the data
MF wrote the paper
AMB, LL and RT reviewed draft manuscript

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