**Molecular Diagnosis of Yeast Infections**

P. Lewis White 1 · Jessica S. Price 1 · Alan Cordey 1 · Matthijs Backx 1

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**Abstract**

**Purpose of Review** The use of molecular tests to aid the diagnosis of invasive yeast infection, in particular invasive candidosis, has been described for over two decades, yet widespread application is limited, and diagnosis remains heavily dependent on classical microbiology. This article will review developments from the past decade in attempt to build on existing knowledge. It will highlight clinical performance and limitations while reviewing developments on recognized procedures; it will also provide insight into novel approaches incorporated in response to clinical demand (e.g. *C. auris* and antifungal resistance) or technological advances (e.g. next-generation sequencing).

**Recent Findings** Limited methodological standardization and, until recently, unavailability of commercial options have hindered the integration of molecular diagnostics for yeasts. The development of certain, novel commercial methods has received considerable evaluation allowing a greater understanding of individual assay performance, but widespread multicentre evaluation of most commercial kits is lacking. The detection of emerging pathogens (e.g. *C. auris*) has been enhanced by the development of molecular tests. Molecular methods are providing a better understanding of the mycobiome, mechanisms of resistance and epidemiology/phylogeny.

**Summary** Despite over two decades of use, the incorporation of molecular methods to enhance the diagnosis of yeast infections remains limited to certain specialist centres. While the development of commercial tests will provide stimulus for broader application, further validation and reduced costs are required. Over the same period of time, *Aspergillus* PCR has become more widely accepted driven by international efforts to standardize methodology; it is critical that yeast PCR follows suit. Next-generation sequencing will provide significant information on the mycobiome, antifungal resistance mechanism and even broad-range detection directly from the specimen, which may be critical for the molecular detection of yeasts other than *Candida* species, which is currently limited.

**Keywords** *Candida* · Yeast · Molecular diagnosis · PCR

**Introduction**

Gaining widespread consensus in the diagnosis of yeast infections has long been a challenge [1]. As the mortality associated with invasive diseases remains high, it is imperative that diagnostic tests are developed and utilized to give both timely and accurate results, enabling effective therapy to be swiftly initiated [2]. Conventional methods of diagnosis, such as microscopy and culture are established essential investigations but lack sensitivity and can delay diagnosis [2, 3]. The current gold standard means of diagnosing invasive disease is blood culture or recovery of yeast from a sterile site (e.g. tissue biopsy) [3, 4]. However, culture takes time to become positive, with accurate identification and susceptibility testing resulting in further delay, which can increase mortality, should effective antifungal therapy be deferred [3, 5]. While empirical antifungal therapy in high-risk patients is frequently administered, it inevitably results in unnecessary overuse of antifungals, increasing expense, risking toxicity and adverse effects and is concerning in an era of increasing antifungal resistance and limited antifungal drug classes [6].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) provides reliable and rapid identification of yeast isolates, but reliance on culture limits clinical utility, preventing a shift from historical therapeutic strategies [7]. Non-culture-based methods, such as PCR,
allow the rapid detection and identification of yeast infections, providing the potential to administer early, effective species-oriented therapy and identify genetic markers associated with antifungal resistance [8]. However, PCR management of yeast infections has had relatively limited clinical incorporation to date [3]. Limited standardization of methods, until recently the lack of commercially available options, and the absence of large-scale clinical trials have likely undermined confidence in appropriateness of non-culture approaches for the diagnosis of invasive yeast infections. Hopefully, clinical trials such as the A-STOP study (ISRCTN43895480) will provide insight into the applicability of molecular tests for the diagnosis of invasive candidosis (IC) and provide data optimal diagnostic strategies, to improve detection of the 50% of cases of IC that are currently missed [9].

This article will review developments from the past decade, in an attempt to build on existing knowledge for an approach that has been in use for over 20 years, but is to gain widespread acceptance. While reviewing developments on recognized procedures, it will provide insight into novel approaches incorporated in response to clinical demand (e.g. C. auris and antifungal resistance) or technological advances (e.g. next-generation sequencing). For a broader review of the subject, please refer to manuscript by the Consortium OPATHY [10*].

Clinical Performance of Candida PCR

The molecular diagnosis of yeast infection has almost entirely focussed on IC, which remains a major cause of sepsis [11]. In 2011, a meta-analysis and systematic review of Candida PCR testing of blood was performed, involving 963 cases of IC in 4694 patients and generating a pooled sensitivity and specificity for the diagnosis of candidaemia of 95% (95% CI: 88–98) and 92% (95% CI 88–95), respectively [3]. It demonstrated that PCR was superior to culture for the detection of proven/probable IC, with the testing of whole blood being superior to testing serum, and specificity of PCR testing increased through sequential positivity.

Given this data, one could question why Candida PCR testing has not gained widespread acceptance. Unfortunately, significant limitations remain; while the performance of PCR for the detection of candidaemia is excellent, blood culture is also a sensitive test when the organism is present in the circulation, and the presence of the organism justifies the molecular testing of whole blood, targeting the intact yeast cell. However, IC is more than just candidaemia, and the optimal specimen choice when testing for IC in the absence of candidaemia remains unclear [9]. Given antigens, such as mannan and (1-3)-β-D-glucan (BDG), are readily detected in serum, it is feasible that cell-free Candida DNA will also be present, and the molecular methods for testing serum/plasma are technically straightforward. Detection of free DNA may be compromised by the methods used to test whole blood. The sensitivity of the T2Candida when testing whole blood was significantly less for deep-seated candidosis (45%) compared to candidaemia (83%); it is not clear whether this reflects the impact of sample choice and subsequent processing, the limited availability of target in the absence of candidaemia or both, but nevertheless the T2Candida outperformed conventional microbiological approaches [12].

The optimal sample choice for the molecular detection of IC remains unclear; will testing whole blood limit the detection of free DNA that may be present in cases of deep-seated candidosis lacking candidaemia, and conversely will PCR testing of serum/plasma underperform for the detection of candidaemia? This uncertainty of optimal sample choice, or understanding of performance limitation according to disease manifestation, highlights the standardization that remains required for Candida PCR to gain widespread acceptance, but this is being addressed through the efforts of the Fungal PCR initiative (www.fperi.eu) and by the availability of commercial Candida PCR kits. To date, the T2Candida is the only commercial platform with extensive clinical validation, and lack of validation limits the incorporation of alternative assays.

As discussed, the performance of PCR for the diagnosis of IC lacking candidaemia is variable. A recent study evaluating the performance of a real-time multiplex PCR for the detection of intra-abdominal candidosis (IAC) when testing serum prior to starting antifungal therapy generated a poor sensitivity of 25% compared to 94% for BDG, although specificity was far superior (91% vs 29%) [13]. Interestingly, 30 cases required blood transfusion before surgery and sampling for Candida PCR, for 25 of these patients, PCR testing of pre-transfusional plasma samples increased sensitivity to 64%, indicating a haemodilution effect on target DNA post-transfusion. It also raises the question of optimal sample type for the molecular detection of IAC. A previous study showed that testing plasma was superior to whole blood for the detection of IC, with no significant difference in performance between serum and plasma, although detection of candidaemia (59%) was lower than the other manifestations (88–89%) when testing serum/plasma [14]. This implies there is no difference between Candida PCR testing of serum or plasma, although significant differences have been noted for PCR detection of invasive aspergillosis, where it was hypothesized that clot formation in serum samples trapped potential target that remained available in plasma [15, 16]. A previous evaluation of the above multiplex real-time PCR generated much improved sensitivity (96%) when testing serum/plasma from intensive care patients predominantly presenting with candidaemia, highlighting the difficulties in diagnosing deep-seated candidosis [17]. Conversely, the MICAFEM study which utilized the same PCR assay demonstrated a poor sensitivity (21–31%) when
testing serum compared to blood or abdominal fluid culture, with samples taken prior to empirical antifungal therapy in ICU patients at high risk of invasive candidosis [18]. It is unclear why sensitivity was so different to the previous study but could reflect the limitations of testing serum (targeting free DNA) when the organism is present. The study also utilized residual blood, with the majority taken for culture, and required both PCR replicates to be positive if a sample was determined positive. Given the low burden of candidaemia (<1 CFU/ml), PCR positivity will typically be in the non-reproducible range (>35 cycles), and therefore, including non-reproducible positives may enhance sensitivity. Indeed, for disease of low incidence, such as IC, excluding disease through a high negative predictive value represents the optimal use of testing; it is therefore unwise and inaccurate to consider samples where one replicate is positive as negative. In the study of Leon and colleagues, also using the same multiplex real-time PCR assay to test ICU patients with severe abdominal conditions, sensitivity was 84%, with Candida PCR considered positive when DNA was detected in a single sample [19]. While specificity was poor (33%), it was improved when PCR positivity was confirmed by another biomarker positivity (e.g. 60% when BDG ≥200 pg/ml). Nevertheless, positive predictive values associated with combined positivity remained significantly <50%, highlighting the difficulty of confirming disease in this cohort.

As indicated by the meta-analysis, the PCR detection of candidaemia is likely to be accurate, but the presence of PCR positivity in blood may not be due to candidaemia, but associated with other forms of IC. It is unlikely that molecular tests will provide a definitive answer for the diagnosis of invasive yeast infections. The results, whether positive or negative, should enhance clinical understanding of the likelihood of infection and whether to treat or withhold therapy based on probability. Strategies for incorporating and interpreting non-culture diagnostics for candidaemia and intra-abdominal candidosis in various at-risk populations have been proposed using post-test predictive values and potential thresholds for action [20].

The use of Candida PCR blood testing could also provide an early indicator of infection, with a murine model demonstrating PCR positivity in blood within 24 h of infective inoculum, albeit for a limited time due to likely renal uptake/infection [21]. The clinical utility of molecular-based testing demonstrated a significant reduction in time to diagnosis and subsequent treatment (31 h) compared to blood culture (67.5 h) for the diagnosis of candidaemia, with PCR-positive patients associated with potentially shorter ICU admissions and lower mortality [22]. The cost-effectiveness of Candida PCR to stop or de-escalate empirical antifungal therapy in ICU patients with suspected fungal peritonitis was demonstrated through a decision tree model, where empirical echinocandin therapy regulated by PCR testing was the most effective strategy, likely to be enhanced when echinocandin costs reduce [23].

A summary of selected non-commercial Candida PCR studies are shown in Table 1.

### Candida PCR Testing in Paediatrics

IC, in particular candidaemia, is a major cause of IFD in hospitalized paediatric patients; although the incidence is declining in the developed world, it remains associated with significant attributable mortality [31]. Molecular tests represent promising diagnostics, but clinical validation is limited, and the smaller sample volumes attainable from paediatrics, especially neonatal patients, may affect sensitivity [32]. In a neonatal study evaluating the performance of the widely evaluated multiplex real-time PCR (described previously), PCR-positive episodes were associated with a trend towards increased mortality and significant increases in multi-organ failure, possibly negated by the use of antifungal therapy [33]. A summary of selected recent studies describing the performance of molecular detection of Candida in paediatrics and neonates, including the T2Candida, is shown in Table 2. Overall performance looks good, but further large-scale studies are required to confirm performance and may be addressed by the BIOPIC trial (NCT02220790) investigating fungal biomarkers for diagnosis and response to therapy for paediatric candidaemia.

### Commercial Candida PCR Assays Other Than the T2Candida

The availability of commercial Candida PCR tests provides methodological standardization, quality control and arguably more extensive, multicentre validation. Many molecular tests have been developed to test positive blood cultures, in doing so providing a rapid and accurate identification similar to direct MALDI-TOF identification [37]. Obviously, the benefits of this approach are limited by the documented confines of blood culture, and for centres with access to a MALDI-TOF, their application will be restricted. The application of commercial Candida PCR testing direct to the specimen is beneficial, and while a range of commercial, Candida PCR assays are available, clinical validation is limited beyond the T2Candida assay [37].

A recent evaluation compared the performance of the Bruker Fungiplex Candida assay with blood culture and the Roche SeptiFast, with the Fungiplex generating excellent sensitivity (100%) and specificity (94%) compared to blood culture when testing whole blood, with sensitivity superior to SeptiFast (60%) [25]. The identification according to the Fungiplex showed good correspondence with culture,
although the authors highlight that not being able to differentiate between the detection of four common causes of IC (C. albicans, C. parapsilosis, C. dubliniensis and C. tropicalis) is a limitation.

The review by Camp and colleagues provides extensive background in to the benefits, limitations and validation of currently available commercial Candida PCR assays or broad-ranging molecular tests with the capacity to detect Candida species [37].

### Table 1  A summary of the performance selected non-commercial Candida PCR studies

| Patient population | Case/control | Sample type | PCR target gene | PCR range | Sensitivity (%) | Specificity (%) | Ref |
|--------------------|--------------|-------------|-----------------|-----------|-----------------|----------------|-----|
| Sepsis             | 10/115       | Blood culture | 18S rRNA | Pan-Candida C. albicans, C. parapsilosis, C. tropicalis, C. glabrata | >99 | 90 | [24] |
| Surgical patients with intra-abdominal candidiasis | 17/39 | Sera or plasma | ITS1 or ITS2 of rRNA | C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. dubliniensis | Post-transfusion fluids = 25% Pre-transfusion fluids = 64% | 91 | [13] |
| ICU/sepsis         | 8/58         | EDTA blood  | 18S rDNA       | Fungiplex C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. dubliniensis | 100 | 94 | [25] |
| ICU                | 5/58         | Sera        | 16S rDNA       | SeptiFast C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei | 60 | 96 |  |
| ICU                | 6/175        | Serum       | ITS1/2 rDNA    | C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii | 16 | 93 | [18] |
| ICU                | 10/39        | Serum       | 18S rDNA       | Fungiplex C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. dubliniensis | 44 | 87 | [26] |
| ICU                | 31/233       | Serum       | ITS1/2 rDNA    | C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii | 84 | 33 | [19] |
| Mammary candidosis | 65/89        | Breast milk | ITS2           | C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. lusitaniae, C. parapsilosis, C. tropicalis | 67 | 21 | [27] |
| Unavailable        | 82 patients  | Whole blood | 5.8S rDNA      | Candida genus | 100 | 98 | [28] |
| ICU/peritonitis    | 23/161       | Peritoneal fluid | Not Stated | Candida genus | 94 | Not provided | [29] |
| Haematology        | 11/61        | Whole blood | 18S rDNA       | Candida genus | 100 | 100 | [30] |
| ICU                | 27/103       | Sera        | ITS1/ITS2 rDNA | C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii | 96 | 97 | [17] |
| ICU with IC        | 55/128       | Plasma/serum| ITS1, ITS2     | C. albicans, C. glabrata, C. krusei, C. tropicalis | 80 | 70 | [14] |

### Table 2  The performance of Candida PCR tests in paediatric populations

| Population                     | Sample type | Assay       | Volume | Case/control | Sensitivity | Specificity | Reference |
|--------------------------------|-------------|-------------|--------|--------------|-------------|-------------|-----------|
| Neonates                       | Whole Blood/Serum/Sterile fluid | In-house | 0.2ml  | 8/147        | 88%         | 82%         | [33]     |
| Paediatrics (including 24 neonates) | Whole blood | In-house | 1–2ml  | 8/46         | 100%        | 89%         | [34]     |
| Paediatrics (including neonates) | Whole blood | T2Candida | 3ml    | 4/59         | 100%        | 95%         | [35]     |
| Paediatrics (no neonates)      | Whole blood | T2Candida  | 2ml (direct application to cartridge) | 15/9 | 100% | 100% | [36] |
Commercial Candida PCR Assays: T2Candida

The T2Candida panel (T2C; T2 Biosystems, Lexington, MA, USA) is an FDA-approved, automated molecular test that detects the five major pathogenic Candida spp. (C. albicans, C. tropicalis, C. glabrata, C. krusei and C. parapsilosis) accounting for >95% of candidaemia, directly in whole blood [38, 39]. The T2Candida system lyses blood cells and Candida cells before performing thermostable PCR amplification of the ITS 2 region using pan-Candida primers and identification/differentiation of C. albicans/C. tropicalis, C. glabrata/C. krusei and C. parapsilosis through hybridization of specific probes and magnetic resonance. Results are generated rapidly (4–5h), requiring limited labour and providing a low limit of detection (LOD) 1–3CFU/ml, dependent on species [38].

While the T2Candida species selection is justified, changes in both the geographic and temporal epidemiology of Candida species causing IC, highlight a potential limitation of the test and individual assays for the detection of emerging Candida species (e.g. C. auris) have been developed (see below). Cases of IC caused by species outside the detection range of the assay should not be excluded when determining assay sensitivity, as clinically they remain a case that was missed by the test, albeit due to design limitations. If these cases are excluded, negative predictive values for the test will be exaggerated, not accounting for the inability of the test to detect other in-frequent causes of IC and the potential for withholding/withdrawing therapy on the basis of a negative T2Candida result be undermined.

The first extensive trial to validate performance of the T2Candida panel to diagnose candidaemia combined the testing of contrived samples and clinical blood compared to blood culture, generating an overall sensitivity and specificity of 91% and 98%, respectively, but clinical cases were limited [40]. The follow-up prospective DIRECT-2 trial 152 patients were enrolled with a diagnostic blood culture performed, of which 36 grew Candida, and the T2Candida sensitivity was 89%, with a specificity of 68% (79/116) [41]. A recent systematic review and meta-analysis of the performance of the T2Candida included eight studies, 2717 patients and 525 cases; it generated pooled sensitivity and specificity of 91% and 94%, respectively [42]. Pooled positive (10.2) and negative (0.08) likelihood ratios were sufficient to confirm or exclude IC when associated with positive or negative results, respectively. While these results are very encouraging, the sensitivity (39%) or specificity (59%, 68%) in some studies are significantly below that demonstrated in meta-analysis [41, 43, 44]. Reduced specificity can be explained by evaluating the performance of novel test that has greater sensitivity over the reference method, resulting in the classification of false positive results associated with the novel test, which could equally reflect false negatives associated with the reference method. Given a major justification for the use of the T2Candida is to prevent the use of the unnecessary antifungal therapy, reports of poor sensitivity are concerning [45]. In the MADRID prospective observational study, sensitivity and negative predictive value were 36% and 80%, respectively, potentially due to the influence of prior empirical antifungal therapy or patients presenting with IC in the absence of candidaemia [46]. Conversely, specificity was high and baseline positivity was a poor prognostic marker.

The performance of the T2Candida in the presence or absence of candidaemia has been recently reviewed in detail [47]. In a study evaluating its performance in 48 ICU patients with (n=18) and without (n=30) intra-abdominal candidosis, the sensitivity and specificity were 33% and 93%, respectively [48]. The T2Candida detected both cases that were blood culture–positive and four additional blood culture–negative cases, confirming the limited rates of detection when the organism is absent from the circulation. Two cases receiving antifungal therapy were also negative. The sensitivity of BDG testing in this cohort was 83%, indicating biomarkers were present in the circulation and detection of free DNA in serum/plasma may have improved molecular detection. Combining T2Candida with BDG generated 100% specificity when both tests were positive and 90% negative predictive value if both were negative [48].

The major restriction to the widespread implementation of the T2Candida platform outside of insurance-based healthcare settings is cost (>$200), which is significantly greater than conventional microbiology and even alternative molecular tests [45]. Obviously, incorporating an internal control to avoid reporting false negative results is essential, but the rate of documented invalid results (5–10%) and the cost per sample reflect a significant unrecoverable, additional expense [40, 41]. This cost of T2Candida testing could be offset by reductions in unnecessary antifungal therapy. An analytical decision tree model was developed to compare antifungal use as directed by T2Candida or blood culture with empirical antifungal therapy [45]. In this model, the T2Candida had the potential to reduce unnecessary antifungal therapy by 98%, but nevertheless, when compared to empirical antifungal therapy, T2Candida diagnostic testing increased cost (approximately $300) and was slightly less effective for the management of suspected candidaemia. This differed to a previous cost-modelling exercise, which demonstrated the T2Candida was both more effective and less costly than empirical therapy, highlighting the limitations of modelling and the need for real-life data [49].

Various recent reviews focus specifically on the T2Candida and should be sourced for a full assessment of the topic [50–53].
Molecular Detection of Candida auris

The emergence of Candida auris as a global multidrug-resistant healthcare-associated pathogen associated with various hospital outbreaks requires the development of rapid and accurate identification methods to optimize infection control measures and restrict the spread of infection, especially during the COVID-19 pandemic [54]. As with the detection of IC, culture has limited sensitivity when testing surveillance or environmental samples, compounded by the delay to result, which is a significant hindrance to timely infection control measures.

Given the problems with misidentification using conventional biochemical methods and the limitations of MALDI-TOF databases lacking C. auris at the time of its emergence, PCR was used as an aid in identifying cultured C. auris. [55–57]. While potentially improving the specificity of identification, the reliance on culture and associated delay hinders infection control, and direct sample testing was needed to fully embrace the benefits of molecular-based identification [58].

The development of a highly sensitive (LOD 1 CFU/PCR reaction) real-time PCR assay for direct testing of patient surveillance swabs and environmental sponges provided detection and significantly increased the environmental detection of C. auris over culture (PCR: 22.5% vs culture 12.4% (difference 10.1%, 95% CI: 6.4–14.1)) [59]. While direct PCR testing of patient swabs did not improve rates of detection (PCR: 19.2% vs culture 20.4% (difference 1.2%, 95% CI: –3.8–13.6)), PCR results for both specimens were available within 4 h or processing compared to 4–14 days for culture. The rapid availability of results is beneficial for limiting unnecessary transmission over time. Importantly, this assay also incorporated an internal control PCR to avoid the reporting of false negative results arising due to inhibition of the PCR process that could also undermine infection control procedures [59]. This C. auris assay, along with others, has now been fully automated using the BD Max Open System, simplifying technical procedures while generating performance comparable to manual assays, and confirmed the high analytical specificity, with no cross-reactivity noted including closely related species (e.g. C. haemulonii and C. duobushaemulonii) [60, 61].

Given C. auris causes a significant degree of invasive disease, particularly fungaemia direct detection from blood samples would be clinically beneficial, and attempts have been made to test serum and whole blood, but further evaluation of clinical performance is required [62, 63].

Various commercial C. auris PCR assays are now available, including the Bruker Fungiplex Candida auris, MycoDART-PCR Candida diagnostic panel, MONODOSE CanAur dde-qPCR and the GENESIG kit that utilizes the primer/probes described by Leach and colleagues, whose performance is described above [59]. Comparison of the OLM AurisID® real-time PCR for direct testing of surveillance swabs without the requirement for DNA extraction with the routine identification using CHROMagar® and MALDI-TOF provided comparable results within a quicker timeframe [64]. Comparison of the OLM and Bruker C. auris assays demonstrated slight differences in LOD and analytical specificity when testing higher concentrations of DNA from species closely related to C. auris [65]. The T2 Candida auris panel provided a fully automated commercial option with performance comparable to culture when testing simulated and clinical axilla/groin swabs [66]. While the development of assays individual to specific emerging species is essential, if the species continues to represent a clinical concern over time, it is important that they are incorporated into broader ranging molecular tests targeting other Candida species, improving cost-effectiveness and clinical utility [67].

The mechanism of C. auris resistance to fluconazole, voriconazole and micafungin has been studied through PCR sequencing of the ERG11 and FKS1 genes [68]. Micafungin-resistant isolates harboured a S639F non-synonymous mutation in hotspot 1 of the FKS1 gene that was absent in susceptible isolates. Of the 36 azole-resistant isolates randomly selected for testing, 12 fluconazole-resistant isolates contained a Y132F mutation, while 24 fluconazole-/voriconazole-resistant isolates contained a K143R mutation in the ERG11 gene. The presence of Y132F and K143R mutations in the ERG11 gene appear definitive for azole resistance, and rapid molecular tests have been developed to detect the Y132F/K143R and S639F mutations in ERG11 and FKS1 genes, respectively [69]. Data linking other mutations with susceptibility profiles are currently conflicting between studies, requiring further large-scale clarification. Whole genome sequencing of C. auris may aid our understanding of the molecular mechanism of resistance and has provided insight into the phylogeny of C. auris, initially identifying the presence of 4 distinct clades (South Asia, East Asia, South America and South Africa), with a potential fifth clade recently identified in Iran [70, 71].

Molecular Detection of Resistance in Candida Species Other Than C. auris

Molecular diagnostics can identify resistance, provided the resistance mechanism has been validated through association with raised or high minimum inhibitory concentrations to a specific antifungal drug, altered drug target, dose-dependent resistance in an animal model or document clinical failures [72•]. In echinocandins, mutations in the 3 FKS genes decrease the sensitivity of glucan synthase to the drug, with resistance dependent on the specific mutation. Fortunately, a small number of FKS mutations are associated with the majority of echinocandin resistance in Candida, and several PCR assays not requiring timely DNA sequencing have been
developed [73–75]. To date, testing has been limited to culture so the clinical utility of such strategies is limited.

Azole resistance in *Candida* species is associated with a wide range of mechanisms, potentially excluding real-time PCR approaches, and while these have been developed, DNA sequencing remains the best option for identifying the mutations associated with resistance, limiting clinical application, particularly direct sample testing [72, 76, 77]. Currently, there are no commercial PCR tests to detect mutations associated with antifungal resistance in yeasts. The development of next-generation (whole genome) sequencing may assist in our understanding/detection of resistance, but direct application to clinical samples will likely require PCR amplification of previously selected genes or regions [78].

**Next-Generation Sequencing in Mycology**

Next-generation sequencing (NGS) represents a revolutionary development in genomic science. In the clinic, much work has focussed on the human genome in order to gain understanding of function and genetic defects that could predispose the individual to certain conditions [10••]. From a microbiological perspective, NGS has helped overcome the limitations of culture when studying the microbiome and mycobiome. Improving our understanding of the composition, anatomical variation, host/microbe and microbe/microbe interaction and imbalance in the microbiome associated with disease development [10••, 79]. On an organism level, antifungal resistance mechanisms and evolutionary/phylogeny of yeast species are being elucidated by NGS [78, 80–83]. Using NGS to analyse RNA transcription can allow us to gain greater understanding of the pathogenicity of fungal species [84], NGS has potential to enhance typing of organism in potential fungal outbreaks.

However, NGS is a complex procedure and is not without limitations in respect to clinical utility. Obviously, complexity is generally associated with delay, which impacts how information generated by NGS can positively impact management of patients with acute disease. Direct application to the specimen will improve on this, but will likely be reliant on PCR amplification of common genes (e.g. ITS regions), but questions over discriminatory power of this approach have been raised, but may be improved by using novel primers [10••, 85, 86]. This approach has been successfully applied to the investigation of corneal scrapes from non-viral infective keratitis [87].

From a practical standpoint, NGS remains costly, requires specific equipment and technical expertise and generates large amounts of data requiring bioinformatics (software and pipelines) for accurate analysis, allowing use and consistency in diagnostic laboratories [10••, 88••]. Technically, optimization of mycological NGS procedures is similar to other molecular-based processes, requiring optimal sampling, awareness and control of fungal contamination, optimal nucleic acid extraction and efficient PCR amplification [88••]. Databases and procedures are somewhat limited in relation mycology and requires significant attention if NGS is to gain widespread clinical use but also utilize the extensive data generated to the maximum [10••].

**Technical Developments**

One of the major issues with using molecular methods to detect fungal pathogens, including yeasts, in blood is the limited fungal burden even in the presence of fungaemia. The testing or larger sample volumes (3–10ml) may help to overcome this problem, but complicate sample processing and nucleic acid extraction methods. Recently, pathogens including *Candida tropicalis* have been concentrated in blood using apolipoprotein H, permitting the use of lower volumes (0.2ml) while maintaining analytical LOD (1CFU/ml) [89]. An alternative approach involved the lysis of large blood volumes while preserving and concentrating microbes (including *C. albicans*) using filters in a small volume that could be used for culture or direct molecular testing [90]. In the initial stage of processing, DNase is added to remove human DNA that can interfere with the process, but also removes potential *Candida* DNAemia target in the patient with IC. Recovery of low fungal burdens (3CFU/ml *C. albicans*) was approximately 50%, so it is unclear if this would benefit the detection of candidaemia where lower burdens are typical [90]. Viscoelastic separation and concentration of *C. albicans* in blood significantly improved real-time PCR detection, providing earlier Ct values and an improved LOD [91].

Obviously, efficient nucleic acid extraction remains critical to successful PCR. If targeting the organism (cell-based DNA), then the fungal cell wall represents a significant hurdle that can limit extraction efficiency. The use of enzymes (Zymolase/lyticase) can be used to remove the fungal cell wall (forming spheroplasts), but recombinant enzymes should be used to overcome possible contamination due to the potential fungal origin of these enzymes. The use of lyticase to detect and identify *C. albicans* in contrived blood did not necessarily improve detection of fungi at the lower concentration, but did improve identification success and limit PCR inhibition [92]. Unfortunately, the fungal burdens included were not necessarily clinically relevant (>10^5 CFU/ml), and at lower levels, the effects could have been more evident. Mechanical disruption (bead-beating) of the fungal cell is an alternative, reducing cost and processing time and likely improving efficiency, although may fracture genomic DNA hindering NGS.

Unlike the molecular detection of *Aspergillus*, attempts to optimize *Candida* PCR have been limited, possibly by early studies demonstrating comparative performance between methods at that time [93, 94]. A recent study compared 11
Molecular Detection of Yeasts Other Than Candida

The development of individual assays specific to species/genera other than Candida is limited by the relatively low incidence of non-Candida invasive yeast infection or the availability of alternative, simple, yet accurate diagnostics (e.g., cryptococcal lateral flow assay). A PCR test specific for the detection of Trichosporon species was described over 20 years ago and more recently a test specific for the black yeast Exophiala dermatitidis in cystic fibrosis patients was reported [123, 124]. While other non-Candida yeast species/genera specific PCR assays likely exist, they remain limited, and it makes more sense to target rarer fungal infections using PCR tests with a broad range of detection, incorporating both Candida and non-Candida yeasts, possibly other filamentous fungi and bacteria. However, it is important that such tests possess the ability to provide a species/genera level of identification, as simply confirming the presence of fungi without identification has limited positive predictive value, compounded by potential contamination and prevents administering species-directed antifungal therapy [125]. If panfungal PCR assays require sequencing to confirm identification, then the delay in getting a result also minimizes the benefit of testing but may utility the testing of histological or tissue biopsies [126].

A recent yeast panel multiplex PCR, not requiring formal nucleic acid extraction, targeting 21 species of Candida, Trichosporon, Rhodotorula, Cryptococcus and Geotrichum, was developed [127]. Initially, it was designed to improve identification of yeasts in the absence of MALDI-TOF, with which it generated 100% concordance when identifying 800 clinical isolates. While this approach is beneficial, providing robustness of identification in resource-limited settings, direct specimen testing would be advantageous, and a multiplex real-time PCR targeting Candida, Aspergillus and Rhizopus generated a sensitivity and specificity of 89% and 100%, respectively, in a pilot study [128].

For the direct, molecular detection of yeasts, especially non-Candida species, pan-fungal PCR approaches likely combined with broad-ranging microbiological detection will most likely provide the optimal diagnostic route. Most of the broad target approaches, including commercial options, simply target common Candida with filamentous moulds and/or a range of bacteria in a single test or simply lack validation for direct specimen testing of unusual yeasts [24, 129, 130]. One approach testing plasma and combining a range of multiplex real-time PCR assays generated good sensitivity for the detection of Candida (85%); specificity was excellent 99.5%, but while the assay targeted a range of fungi (Aspergillus sp., Mucorales sp., hyalohyphomycoses and endemic fungi), yeast detection was limited to C. albicans, C. glabrata and C. krusei [130].

Unfortunately, systems using PCR/electrospray ionization-mass spectrometry (e.g., Abbott IRIDICA), with the capacity to replicate the range of detection provided by culture, but with greater sensitivity when directly testing a range of samples, appear to be currently unavailable [131–135]. Other broad-ranging approaches (e.g. Luminex xTAG technology) only have limited evaluation, and developments appear to have stalled [136]. Subsequently, the molecular detection of yeast species other than Candida requires significant development, which is unlikely to get.

Concluding Remarks

The impact of molecular diagnostics for invasive yeast infections has been limited by a lack of standardization and commercial assays. With both being addressed, it is hoped that molecular testing will become routine practice, making the potential benefits widely available. The emergence of multidrug-resistant species (i.e. C. auris) has demonstrated how advantageous molecular testing can be for the diagnosis and emergence of novel species. NGS will be essential in gaining an understanding
of the molecular mechanisms of resistance in established yeast pathogens and identifying potential new antifungal targets. Unfortunately, beyond Candida species, the use of specific molecular testing is limited by the low incidence of that particular disease, and potential methods (PCR/electrospray ionization-mass spectrometry) with the capacity to replicate the detection range of culture appear no longer widely available. With an
ever-increasing population at risk of yeast infections, the range of potential pathogens will undoubtedly evolve, and it is essential that technology be embraced to ensure an accurate and timely diagnosis.

**Declarations**

**Ethics Approval**  Not required

**Conflicts of Interest**  P. Lewis White: Performed diagnostic evaluations and received meeting sponsorship from Associates of Cape Cod, Bruker, Dynamiker and Launch Diagnostics; speakers’ fees, expert advice fees and meeting sponsorship from Gilead; and speaker and expert advice fees from F2G and speaker fees from IMMY, MSD and Pfizer. Is a founding member of the European Aspergillus PCR Initiative. Mathijs Backx: Speakers’ fees, expert advice fees and meeting sponsorship from Gilead. Meeting sponsorship from AbbVie.

Jessica S. Price and Alan Cordey declare that they have no conflict of interest.

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- Of major importance

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