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Evaluation of the Commercial Rapid Trehalose Test (GLABRATA RTT) for the Point of Isolation Identification of *Candida glabrata* Isolates in Primary Cultures

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Abstract Candidaemias account for 10–20% of nosocomial bloodstream infections depending on the study. Whilst *Candida albicans* remains the most frequently isolated species, *Candida glabrata* may be responsible for as many as 10–25% of all candidaemias. Moreover, *C. glabrata* is generally less susceptible to the azole antifungals than the majority of other pathogenic yeast species. Thus, a rapid test for the specific identification of isolates of *C. glabrata* would be useful for patient management if it could be performed at point of isolation, on primary cultures grown on standard mycological media directly from patient specimens. Under certain conditions, *C. glabrata* rapidly hydrolyses trehalose into glucose. The GLABRATA RTT kit allows detection of the preformed enzyme responsible for this action. This study has assessed GLABRATA RTT as an identification tool specifically at point of isolation. Sixty test isolates were evaluated: 39 clinical isolates of *C. glabrata* identified at the UK Mycology Reference Laboratory, examples of the recently described genetic relatives of *C. glabrata*, *Candida nivariensis* ($n = 6$) and *Candida bracarensis* ($n = 1$), and a selection of other common pathogenic yeast species ($n = 14$). The test provided results within 30 min. Although 77% (30/39) of confirmed *C. glabrata* isolates were correctly identified by GLABRATA RTT (positive trehalase test), 23% (9/39) of isolates gave negative or equivocal results. All other yeast species gave negative results. The performance of GLABRATA RTT in this study is compared to previous evaluations of the test which employed isolates pre-cultured on specialised media and to other existing conventional identification methodologies.

Keywords *Candida glabrata* · Identification · Rapid trehalose test · Candidaemia · Point of isolation identification

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Introduction

Invasive fungal infections caused by *Candida* spp. remain a significant cause of mortality in immunocompromised patients and those undergoing invasive procedures (reviewed in [1, 2]). While *Candida albicans* remains the most common *Candida* species encountered in human infections, *Candida glabrata* is frequently isolated from both mucosal and systemic infections worldwide [1–4]. Indeed, estimates suggest that *C. glabrata* comprises 5–25% of yeast isolates in the clinical setting and might be the causative agent in as many as 10–25% of all candidaemias [3]. Moreover, numerous studies have indicated that *C. glabrata* as a species is generally less susceptible to the azole antifungals, particularly fluconazole, than most other

yeast species encountered in the clinical setting (reviewed in [3]). The rapid and accurate identification of this species can therefore have a direct impact upon patient management [5].

Current methods for identifying *Candida* species are many and varied [6–13]; in a clinical setting, the most common method may be automated as with the bioMérieux VITEK 2 card system (bioMérieux VITEK, Marcy l'Etoile, France) or by the use of commercial sugar assimilation kits or auxanograms such as Auxacolor 2 (Bio-Rad). Whilst most of these methods have high sensitivity and specificity, they can require an incubation period of 24 h or more. Furthermore, it may be impossible to distinguish *C. glabrata* from its close genetic relatives *C. nivariensis* [15] and *C. bracarensis* [16] with such tests [17].

The need for a specific *C. glabrata* identification tool has driven the development of the rapid trehalose test from a simple biochemical test to a commercially available kit-based system [18]. *C. glabrata* may, in certain conditions, rapidly hydrolyse trehalose into glucose [4], and this has been used as a method for rapid identification of this organism [14, 19]. GLABRATA RTT is a kit-based system manufactured by SERFIB (Paris, France) distributed by Fumouze Diagnostics (Levallois-Perret, France), which is designed to detect within 30 min the preformed enzyme, trehalase, responsible for hydrolysing trehalose into glucose. For the cryptic species *C. nivariensis* and *Candida bracarensis*, which require molecular techniques to confirm identification [15–17], *C. nivariensis* has been shown to lack the ability to assimilate trehalose on auxanogram, whereas *C. bracarensis* has demonstrated a variable ability to hydrolyse trehalose [16]. Isolates of *C. tropicalis* may also breakdown trehalose; however, unlike *C. glabrata*, *C. tropicalis* will assimilate maltose, and so this sugar is included in the kit to help reduce false-positive results. Most previous studies aimed at evaluating this system have reported excellent sensitivities and specificities [20–22]. However, several reports suggested that the composition of media employed for primary culture might influence the ability of GLABRATA RTT to correctly identify *C. glabrata* isolates, with the best results obtained particularly when primary isolates had been cultured on specific (particularly chromogenic) media [20–22, 24]. Since the majority of microbiology laboratories in the UK isolate pathogenic yeasts by culture (typically for 24–48 h) on a variety of standard microbiological and/or mycological media (principally Sabouraud's agar), the potential clinical utility of GLABRATA RTT (the extremely rapid identification of "primary" *C. glabrata* isolates) would be reduced if all yeast isolates had first to be sub-cultured for a further 24–48 h on secondary media prior to testing. Here we have evaluated the ability of GLABRATA RTT to correctly identify isolates of *C. glabrata* grown on standard mycological media (Sabouraud's agar) using a panel of clinical isolates that have been reliably identified using conventional and molecular methods at the UK Mycology Reference Laboratory (MRL).

Materials and Methods

Test Isolates and Conventional Phenotypic Identification

The isolates included in the current study comprised 33 clinical isolates of *C. glabrata* that had been unambiguously identified at the MRL, 5 reference strains of *C. glabrata* from the National Collection of Pathogenic Fungi (NCPF) housed at the MRL (isolate identifiers NCPF 8875 through 8879), confirmed reference isolates of *C. nivariensis* ($n = 6$; NCPF 8845, 8846, 8848, 8850, 8853, 8862) and *C. bracarensis* ($n = 1$; NCPF 8894) and recent clinical isolates of several other yeast species including *C. albicans* ($n = 3$), *Candida parapsilosis* ($n = 4$), *Candida lusitaniae* ($n = 2$), *Candida krusei* ($n = 1$) and Saccharromyces cerevisiae ($n = 1$). Three mixed cultures (each containing *C. glabrata* plus *C. albicans*) were also included. Isolates were grown at 37°C for 24–96 h on Sabouraud's media (Oxoid). When necessary, isolates were also cultured on chromogenic agar (CHROMagar; Mast technologies), for 48 h at 37°C following the manufacturer's recommendations. Isolates from the NCPF were subcultured at least twice before testing. The identity of all isolates was confirmed at the MRL by subjecting them to our routine laboratory identification protocol which includes, Germ Tube Test [6], Auxacolor 2 (Bio-Rad), API C20 AUX (bioMérieux-VITEK) and if necessary, in-house molecular identification as previously described [17].

GLABRATA RTT Test

The GLABRATA RTT test format is a plastic card with four groups of three wells containing dehydrated sugar solutions of trehalose, maltose and a basic medium. Test isolates were subjected to the GLABRATA RTT test exactly as described in the manufacturers' instructions. Briefly, a suspension of each isolate was prepared in 2 ml sterile H_2O, to a turbidity of approximately 5 McFarland standard. Each of the three wells on an RTT test card was then inoculated with 25 µl of the resulting suspension, and the cards were incubated at room temperature (~18°C) for 10 min. After this time, 25 µl of Active Reagent was added to each well, and test cards were subjected to a further incubation of between 5 and 10 min at room temperature. According to the manufacturers' instructions, a "more or less" dark orange colour is scored as a positive reaction, whereas no colour change in the test wells should be interpreted as a negative reaction. Results where the test wells did not appear colourless but lacked sufficient pigmentation to be read as orange (or purple, if stopped with 3 M H_2SO_4) were deemed not interpretable, or equivocal. No guidance is provided with the kit insert regarding this, so for the purpose of this study, they were considered to be a negative result. Values for positive and negative predictive values (PPV and NPV, respectively) and sensitivity and specificity were calculated according to standard formulae (PPV = true positives/true positives + false positives; NPV = true negatives/true negatives + false negatives; specificity = [true negatives/true negatives + false positives] × 100; sensitivity = [true positives/true positives + false negatives] × 100).

Results

The GLABRATA RTT kit correctly identified 30/39 (77%) of the confirmed *C. glabrata* isolates included in this study (Table 2). However, correct identification (a positive trehalase test) was not always achieved with isolates that had been subcultured and grown for only 24 h prior to testing (data not shown). Indeed, results were generally clearer to read with isolates that had been grown for at least 48 h. Results appeared more visible after 20-min initial incubation, before addition of the active reagent, rather than the manufacturer recommended 10 min (data not shown). Stopping the reaction using 3 M H_2SO_4 also provided a more solid interpretation of colour change (Blue/Grey to Purple), although this is somewhat operator subjective.

Table 1 GLABRATA RTT results and organisms tested

Organism	RTT+	RTT−	Total
C. glabrata	30	9	39
C. nivariensis	0	6	6
C. bracarensis	0	1	1
Other yeast species	0	11	11
Mixed (*C. glabrata* plus *C. albicans*)	0	3	3

RTT rapid trehalose test

The GLABRATA RTT system failed to identify the remaining 9 *C. glabrata* isolates (23%) that were subjected to testing (Table 1), despite repeated testing. These GLABRATA RTT-negative *C. glabrata* cultures remained negative even when grown for 96 h prior to repeat testing or when subcultured for 48 h on chromogenic agar (CHROMagar; Mast technologies) prior to testing. Since GLABRATA RTT is recommended for use on primary isolation cultures, three mixed cultures were also included in the current study (Table 1). They had been isolated directly from clinical specimens, and each contained a mixture of *C. glabrata* and *C. albicans*, with *C. glabrata* as the majority organism in mixtures A and C. The GLABRATA RTT system failed to detect the presence of *C. glabrata* (on the basis of a positive trehalase test) in any of the three mixtures (Table 1).

Finally, it should be noted that there were no false-positive results obtained with the 21 non-*C. glabrata* isolates tested in the current study, including the 6 isolates of *C. nivariensis* available for study. Although some strains of *C. bracarensis* may be able to hydrolyse trehalose and thereby give a false-positive result, this was not the case with the single clinical isolate that was available for inclusion in this study. However, there is currently little clinical evidence to suggest it is necessary to distinguish this organism from *C. glabrata* in a clinical setting, so the significance of this is unclear [15].

Discussion

There is a growing need for rapid identification of yeasts due to the rise in non-*albicans* species causing disease in increasingly immuno-suppressed patient

populations [1–4]. A rapid identification can provide vital information resulting in prompt and targeted therapeutic options [4]. Previous studies have evaluated the GLABRATA RTT system as an extremely rapid ID system specifically for isolates of *C. glabrata* [20–22]. Although this test can yield results in 30 min, test performance was greatly affected by the composition of the primary media on which the isolates had been cultured, and optimal performance required either the use of specific formulations of Sabouraud's media or the additional subculture of isolates for 1–2 days on chromogenic agar [20–22]. In the UK, most laboratories routinely culture suspected pathogenic yeasts (from blood cultures, tissues or other fluids) on standard microbiological media, which might include Sabouraud's agar. Thus, in the current study, we assessed the performance of the GLABRATA RTT system as a point of isolation test, using isolates that had been cultured on Sabouraud's agar.

Under these conditions, the GLABRATA RTT system was not able to identify 9/39 (23%) confirmed isolates of *C. glabrata.* Since GLABRATA RTT is dependent upon the production of preformed trehalase enzyme, it is possible therefore that an insufficient amount of this enzyme is produced by some isolates after only 24 h of culture. However, those isolates that gave negative results after 24 h of culture did so consistently even after 96 h of growth. Very similar, elevated FNRs have been reported previously using GLABRATA RTT on isolates grown on Oxoid Sabouraud's agar [21], using dipstick trehalase detection systems and certain, but not other preparations of mycological media [23–25].

In the light of these previous reports, and data suggesting that test performance was optimal using cultures that had first been grown on chromogenic agar [20–22], these 9 isolates were subcultured for 2 days on CHROMagar prior to retesting. All 9 isolates still remained negative, suggesting that the failure of GLABRATA RTT to identify these strains was not simply due to inappropriate choice of culture media. A possible alternative explanation is that the sensitivity of GLABRATA RTT is insufficient to correctly identify certain UK strains of *C. glabrata* that might constitutively produce low levels of trehalase. It should be noted that the identity of these GLABRATA RTT-negative *C. glabrata* isolates was confirmed by both rDNA sequencing and retesting with Auxacolor 2 and morphological examination (data not shown). Interestingly, all isolates gave typical *C. glabrata* profiles with Auxacolor 2, demonstrating that they still possessed the ability to assimilate trehalose when it was the sole carbohydrate source.

Similarly, although GLABRATA RTT is designed to be used at point of isolation, when it is not always clear whether a culture is mixed or not, the kit failed to detect the presence of *C. glabrata* in 3/3 mixed cultures subjected to testing. On the basis of subculture on chromogenic agar, *C. glabrata* was the majority organism in 2/3 mixtures.

The calculated specificity and the false-positive rates (FPR) of GLABRATA RTT are encouraging and compare reasonably with many of the other, currently employed phenotypic and molecular identification tools (Table 2). However, the elevated false-negative rates obtained with GLABRATA RTT and isolates grown on certain primary culture media (this report; [21]) argue against the use of this system as a point of isolation identification methodology. Previous studies have evaluated other commercial and in-house platforms designed to specifically and rapidly identify *C. glabrata* isolates on the basis of trehalose fermentation or assimilation ([14]; Table 2). Significantly, several of those alternative systems exhibited elevated rates of false positivity (with non-*C. glabrata* species), and all platforms evaluated failed to identify a proportion of *C. glabrata* isolates that were tested [(23–25]; Table 2 and references therein). It can be predicted that both false-positive and false-negative results would have (albeit different) implications for patient care. It is unlikely that a testing laboratory would further pursue the identification of any isolate that yielded a positive result, leading to misidentification of some isolates as *C. glabrata* and the possibility that those patients would receive perhaps more costly antifungal agents and intravenous therapy when an oral azole antifungal would have been appropriate. Conversely, although it is probable that isolates that were falsely scored as negative would be further identified to species level using additional platforms, it is also likely that those patients involved might receive inappropriate standard dose fluconazole therapy in the intervening period prior to correct identification.

In summary, although GLABRATA RTT (and certain other trehalose-based *C. glabrata* ID kits) are more rapid than certain current general yeast identification systems (Table 2), adequate test performance requires the careful choice of primary culture media, and many of the current alternative systems are able to accurately identify in

Table 2 Comparison of GLABRATA RTT with other current identification methodologies for identifying *C. glabrata*

Test	Time (h)	Cost (£)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Data
GLABRATA RTT (Fumouze Diagnostics)	0.5	3.77	77	100	100	66.7	This study
Rapid assimilation trehalose broth (Mayo Clinic)	1	0.03	96.6	74.1	97.5	66.7	[14]
Rapid assimilation trehalose broth (Remel)	3	1.03	91.5	96.3	99.6	50.9	[14]
Yeast fermentation broth (Remel)	24	2.67	95.0	89.0	98.9	36.8	[14]
Trehalose fermentation broth (Hardy diagnostics)	24	0.71	96.0	100	100	30.7	[14]
Auxacolor 2 (Bio-Rad)	24[a]	3.72[b]	100	100	100	100	[13, 17]
RapID Yeast (Innovative Diagnostics Systems, Norcross, Ga.; distributed by Remel Inc)	4	6.66[c]	95.5	99.2	100	99.2	[7[d], 8, 9[d], 12]
Vitek 2 YST (bioMérieux VITEK)	18	1.90[e]	96	99.4	99.8	99.4	[7[d], 10, 11]
API 20C AUX (bioMérieux-VITEK)	72	4.20	97.1	99.4	100	99.1	[8, 9, 12]
Pyrosequencing® (Biotage-Qiagen)	6	1.49[e]	100	100	100	100	[20, 21]

The *C. glabrata* specific tests are in bold. For the other general yeast identification tests, values are calculated for identification of *C. glabrata* isolates

PPV positive predictive value, *NPV* negative predictive value

[a] *C. glabrata* identified at 24 h, other yeasts may require up to 72 h

[b] Including cornmeal agar with tween 80 plate essential for complete profile

[c] Includes kit and reagents

[d] Identification required inclusion of supplementary tests

[e] Excludes initial machine costs

excess of 90% of all clinical yeast isolates, comprising many different species (see for example [12, 17, 26–29]) with comparable, or lower, cost per test (Table 2). Moreover, although their implementation in smaller microbiology laboratories may be hindered by elevated set-up and machine costs, recent advances in rapid molecular techniques such as pyrosequencing [26, 27] and novel biophysical methods employing mass spectrometry [28, 29] may remove the usefulness of biochemical "bench-top" testing altogether.

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