Clinical isolates and laboratory reference Candida species and strains have varying abilities to form biofilms

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Abstract
Candida biofilms are a major virulence trait for this yeast. In this study, the biofilm-forming ability of the major medically important clinical and laboratory reference strains was compared. Biofilms were quantified using traditional methods, that is, crystal violet (CV), tetrazolium (XTT) reduction and colony-forming unit assays (CFU), and two new methods: an automated cell counter (ACC) and biofilm suspension turbidity (BST) method. Biofilms could be categorized based on biofilm biomass (high, medium and low) and growth state (high and low). Candida albicans genotypes, A, B and C, showed medium biofilm mass and low growth rate, and only one Candida albicans laboratory strain, ATCC MYA-2719, matched this biofilm category. Of all non-albicans Candida species tested, only Candida dubliniensis and Candida glabrata laboratory and clinical isolates had similar biofilm development. The ACC and BST methods for measuring biofilm significantly correlated with CV and CFU biofilm mass measurements. Thus, biofilm mass can be rapidly assessed using biofilm disruptive/cellular nondestructive methods allowing yeast biofilm cells to be used for further analysis. In conclusion, Candida laboratory reference strains and clinical isolates have been shown to form biofilms at different rates; hence for validity, the selection of laboratory reference strains in biofilm studies may be critical for virulence assessment.

Introduction
Biofilms can be defined as microbial communities of surface-attached cells encased in a self-produced matrix of extracellular polymeric substance (Donlan & Costerton, 2002). They are formed in response to a variety of environmental and physical cues such as high cellular density, nutrient deprivation and physical stresses (O'Toole et al., 2000). Their self-contained and protected environment allows biofilms to act as a nidus of organism dissemination and an ongoing source of infection (Chandra et al., 2008). In nature, biofilms represent the most prevalent type of microbial life and are deemed as crucial to the development of clinical infections (Davey & O'Toole, 2000). They can be formed by pathogenic bacteria and fungi and are thought to be a major factor in the persistence of these organisms on inert surfaces of indwelling devices such as catheters, joint replacements, dentures and prosthetic heart valves, as well as natural host surfaces (Tunnet et al., 1996; Donlan, 2001).

The fungus Candida is a member of the commensal microbiota and inhabits approximately 30–40% of clinically healthy mouths (Arendorf & Walker, 1980). Under certain circumstances, particularly in immuno-deficient patients such as patients infected with HIV/patients with AIDS and organ transplants recipients, Candida can cause a variety of opportunistic infections ranging from superficial mucosal infections to life-threatening systemic diseases (Samaranayake & MacFarlane, 1990). Candida is well known for its ability to form biofilms that are recognized as a major virulence attribute for this yeast (Costerton et al., 1995; Douglas, 2003). Of importance, the biofilm cells are phenotypically distinct from their planktonic cells and exhibit elevated resistance to host defences...
and administered antifungal agents (Baillie & Douglas, 2000; Samaranayake et al., 2002). It has been estimated that 65% of all hospital infections originate from these microbial aggregates (Mah & O'Toole, 2001). Due to their clinical significance, researchers are now recognizing the value of investigating the biofilm communities rather than planktonic forms when characterizing the pathogenic potential of microorganisms (Silva et al., 2010).

So far, a variety of model systems have been described for the in vitro study of Candida biofilms growth and development. Biofilms are generally grown on inert substrates over several days, and the resultant biofilm mass and metabolic activities are measured. The colony-forming unit (CFU) assay, a resource/labour-intensive and slow method, is commonly used to measure biofilm mass and is undertaken after biofilm disruption (Donlan & Costerton, 2002). Over the last decade, several surrogate assays for biofilm quantification in microtiter plates have been described. These include quantification of biofilm mass via crystal violet (CV) or syto9 stain assays; specific staining of biofilm matrix by 1,9-dimethyl methylene blue dye; and biofilm viability assays for quantification of viable cells by resazurin, fluorescein diacetate or tetrazolium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) salt (Peeters et al., 2008). The latter has been used as a measure of biofilm development, viability, growth and metabolic activity as the XTT salt is reduced to formazan by the metabolically active cells and is considered as highly reliable test for indirect quantification of biofilm mass (Chandra et al., 2001; Ramage et al., 2001; Adam et al., 2002; Tumbarello et al., 2007).

It has been shown that biofilm-forming ability and structure are microbial strain- and species-dependent (Silva et al., 2009). Furthermore, phenotypic and genotypic studies have revealed a relationship between Candida albicans genotype and virulence properties (Zeng et al., 2008; Sardi et al., 2012). For example, in a recent study, genotypes A and B of C. albicans were identified in subgingival biofilms of diabetic patients with higher cell surface hydrophobicity reported for genotype A (Sardi et al., 2012). This would indicate that certain C. albicans strains have greater propensity to cause superficial or deep infections than do other strains. Thus, evaluating the variations in fungal biofilm formation will have important clinical and therapeutic value. So far, no comparative studies involving biofilms of the major medically relevant Candida species and strains have been conducted. In addition, no study has placed emphasis on the differences in biofilm-forming ability of clinical and laboratory reference strains of the same Candida species. The number of passages that laboratory reference Candida strains have undergone and the well-known genomic plasticity of this yeast (Selmecki et al., 2010) may raise questions about the choice of laboratory reference strains for the study of clinically relevant biofilm formation and other virulence characteristics.

Therefore, the aim of the present study was to compare biofilm-forming ability of different clinical and laboratory reference Candida species and strains seeded at different cell inocula. Biofilms were quantified using traditional methods, that is, CV, tetrazolium (XTT) reduction and CFU assays. Additionally, two biofilm disruptive/cellular nondestructive methods to rapidly assess biofilm mass with one measuring biofilm suspension turbidity (BST) and the other automatically counts cells of the resuspended biofilms using an automated cell counter (ACC) were assessed.

**Materials and methods**

**Organisms and growth conditions**

A total of 22 Candida strains were used in the study. Nine were clinical isolates including C. albicans genotypes (A, B and C), Candida parapsilosis, Candida tropicalis, Candida glabrata, Candida dubliniensis, Candida guilliermondii and Candida kruzei, which had been previously recovered from oral infections in HIV-infected patients. The other 13 Candida strains were obtained from the American Type Culture Collection (ATCC) and include the following: C. albicans CBS 562 (ATCC 18804), C. albicans (ATCC 32354), C. albicans (ATCC 90028), C. albicans (ATCC 90234), C. albicans LGH 1095 (ATCC MYA-2719), C. albicans SC5314 (ATCC MYA-2876), C. glabrata CBS 138 (ATCC 2001), C. parapsilosis (ATCC 22019), Candida lusitaniae IFO 1019 (ATCC 34449), C. kruzei (ATCC 6258), C. guilliermondii (ATCC 6260), C. tropicalis (ATCC 750) and C. dubliniensis CBS 7987 (ATCC MYA-646). All Candida strains were subcultured from thawed suspensions of pure clinical isolates and ATCC stock cultures. The identity of all strains was confirmed by PCR amplification and restriction fragment polymorphism analysis of the intergenic transcribed spacer regions of rDNA as previously described (McCullough et al., 1999). For determination of clinical C. albicans genotypic subgroups, PCR using primers that span the transposable introns in the rDNA was performed as previously described (McCullough et al., 1999).

Prior to each experiment, the yeasts were streaked diluted on Sabouraud dextrose agar (SDA; Department of Microbiology and Immunology, Media preparation unit, The University of Melbourne, Melbourne, Vic., Australia) and incubated at 37 °C for 48 h and a single colony isolated. A large loop of actively growing cells was transferred to sterile yeast–peptone–glucose (YPG) broth [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose (Department of Microbiology and Immunology, Media
preparation unit, The University of Melbourne) and incubated for 18 h at 37 °C. Cells were harvested by centrifugation at 750 g for 5 min and washed with phosphate-buffered saline (PBS; Sigma-Aldrich, NSW, Australia). The cell pellet was resuspended in YPG broth and the cell concentration adjusted, after counting using an improved Neubauer hemocytometer, to $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^7$ cells mL$^{-1}$.

**Biofilm formation**

*Candida* biofilms were grown according to previously published protocol (Jin et al., 2003; Silva et al., 2010). Biofilms were produced in the commercially available pre-sterilized, polystyrene, flat-bottomed 96-well microtiter plates (Nunc, Denmark). Briefly, 200 µL of standard cell suspension of $1 \times 10^3$, $1 \times 10^4$ or $1 \times 10^5$ cells mL$^{-1}$ prepared as above was inoculated into selected wells and incubated at 37 °C for 72 h in an orbital shaker (Alyos, Thermo Fisher scientific, NSW, Australia) at 90 r.p.m. The growth media were replenished every 24 h, by removing 150 µL and replacing it with fresh media (150 µL). Experiments were performed in triplicate on two separate occasions for each strain with uninoculated controls.

**Biofilm growth measurement**

Five methods were used to quantify biofilm formation: CV staining was used for assessing the biofilm mass; XTT salt reduction, to determine the *in situ* total biofilm metabolic/growth activity, whereas the number of cultivable cells was established by the CFU counts. The assays were performed as previously described (Jin et al., 2004). Two additional assays were developed during the study, to assess biofilm mass, using an ACC assay (Bio-Rad, NSW, Australia) and BST assessment using NanoDrop spectrophotometer (Thermo Fisher scientific). The main comparisons in biofilm-forming ability between the 22 *Candida* species and strains were based on CV and XTT assays; the two standard assays were used to measure biofilm mass and metabolic activity, respectively. To test the applicability of the two new assays for measuring biofilm mass, the biofilm formation was further assessed using the ACC and BST assays with the CFU method as a gold standard method. All isolates were assessed in triplicate and repeated on two separate occasions. Correlation between all above assays was measured.

**CFU assay**

After biofilm formation, the supernatants were removed and biofilms washed twice with 200 µL of PBS. A planktonic cell suspension of the washed biofilms was achieved with 200 µL of PBS and mechanical disruption using a pipette, and the cell suspensions were then vortexed for 10 s to further disaggregate cell clusters. The cell suspensions were serially diluted and inoculated on SDA. Agar plates were incubated for 48 h at 37 °C in incubator (Forma Direct Heat CO$_2$ incubator; Thermo Fisher scientific), and the CFUs per individual biofilms were enumerated (Silva et al., 2010).

**XTT reduction assay**

The XTT (Sigma-Aldrich) reduction assay was used to determine the *in situ* biofilm growth activity. After biofilm formation, the culture medium was aspirated and nonadherent cells removed by washing the wells twice with 200 µL of PBS. XTT stock solution was prepared by reconstituting the XTT with 1% phenazine methosulphate mixture (Sigma-Aldrich) in filter-sterilized PBS at concentration of 1 mg mL$^{-1}$. Forty microlitre of the above solution and 160 µL of PBS were added to each well of the microtiter plate containing prewashed biofilms to make up a total volume of 200 µL of PBS-XTT–phenazine methosulphate solution in each well. The plates were then incubated at 37 °C for 3 h in the dark. Following incubation, 150 µL was aspirated from each well, transferred to individual 1.5-mL plastic tubes and centrifuged at 750 g for 5 min, and 100 µL was transferred to a fresh 96-well microtiter plate. A microtiter plate reader (Victor$^3$, 1420 multilabel counter; Perkin-Elmer, NSW, Australia) was used to measure the absorbance at 450 and 620 nm. Measurement at a reference wave length of 620 nm was subtracted from the 450 nm measurement to remove background fluorescence. Furthermore, control wells with no biofilms were used for each experimental assay to assess contamination.

**CV staining assay**

The CV assay was undertaken as previously described (Peeters et al., 2008). The formed biofilms were washed twice with 200 µL of PBS. Washed biofilms were fixed by adding 200 µL of methanol to each well (15 min), after which the supernatants were removed, and the plates were air-dried for 45 min. Subsequently, 200 µL of a 0.1% (w/v) CV solution was added to each well and incubated at room temperature for 20 min, after which excess CV solution was removed by washing the plates gently under running tap water. Two hundred microlitres of 33% (v/v) acetic acid (destaining solution) was then added to the wells to release the bound CV. Finally, 100 µL of this destaining solution was transferred to a fresh 96-well microtiter plate and the absorbance determined using a microtiter plate reader (Victor$^3$, 1420 multilabel counter; Perkin-Elmer) at wave length of 620 nm.
ACC assay
TC10 ACC (Bio-Rad) was used to count the cellular component of the formed biofilms. This counter is a benchtop instrument that counts mammalian cells utilizing microscopy with autofocus and an image analysis algorithm. Total cell count (with or without trypan blue staining) can be obtained to assess for cell viability via trypan blue exclusion. However, in the current study, total biofilm cell mass was measured without discrimination between live and dead cells. The biofilm formed at the bottom of each plate was treated in exactly the same manner as was for the CFU assay to create a planktonic cell suspension. The cells from the resuspended biofilms were counted by the TC10 ACC according to the manufacturer’s instructions. Biofilm cell suspensions were diluted as necessary to be within the detectable cell concentration range of the counter, and the resultant cell count was multiplied by the dilution factor.

BST assay
NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher scientific) was used to evaluate BST. After disruption of the washed biofilms as outlined in the CFU assay to form a planktonic cell suspension, the biofilm was diluted to 1 mL and optical density (520 nm) of the cell suspension determined using the cuvette (path length of 1 cm) installed in the NanoDrop 2000/2000c Spectrophotometer according to the manufacturer's instructions.

Statistical analysis
Results were analysed using SPSS software (PASW Statistics GradPack 18 Inc.). One-way analysis of variance (ANOVA) was performed to compare the results at the three different initial cell concentrations by applying Levene's test of homogeneity of variance and the HSD Tukey's multiple comparisons. Pearson correlation analysis was used to assess the degree of correlation between biofilm assays. Results were considered statistically significant when $P < 0.05$.

Results
Evaluation of biofilm mass quantification methods
Two additional biofilm mass-quantifying assays were developed using an ACC method and a BST method, and the assays’ reliability was compared by correlating them with number of CFU. In the first set of experiments, nine Candida strains were grown as biofilms (starting inocula of $1 \times 10^7$ cell mL$^{-1}$). After 72 h, biofilm mass was determined by CV staining and CFU (the gold standard method) and compared with ACC and BST methods (Tables 1 and 2). Correlation analysis showed that there was no significant difference in determining biofilm mass using ACC and BST compared with CFU or CV. Interestingly, both ACC and BST showed higher correlation ($r = 0.874$ and $r = 0.811$, respectively) with CFU than the CV method ($r = 0.726$; Table 2). Therefore, both new assays were used to further analyse biofilm mass of all Candida species and strains used in this study.

Effect of initial cell concentration on biofilm-forming ability of clinical isolates and laboratory reference C. albicans and Candida species
A total of 22 Candida isolates were assayed for biofilm formation after 72-h incubation under different experimental initial cell concentrations (Figs 1 and 2). The Candida isolates all formed biofilms in the 72-h assay, but the level of biofilm development was dependent on the starting inocula used. With the increase in initial cell inocula, 15 of the Candida isolates showed significantly ($P < 0.05$) higher biofilm mass (as measured by CV), and twelve Candida strains formed significant ($P < 0.05$) actively growing (as measured by XTT) biofilms when a $1 \times 10^7$ cell mL$^{-1}$ starting inocula was used compared with a $1 \times 10^3$ and/or $1 \times 10^4$ cell mL$^{-1}$ starting inocula (Figs 1 and 2). Similarity in biofilm mass was observed at a starting inoculum of $1 \times 10^7$ cell mL$^{-1}$ between C. albicans clinical (A–C) and laboratory reference strains ATCC 18804, 90028 and ATCC MYA-2719 and between the clinical and laboratory reference non-albicans Candida strains, C. krusei and C. guilliermondii. However, at starting inocula of $1 \times 10^7$ cell mL$^{-1}$, only laboratory reference C. albicans strains ATCC 90028 and ATCC MYA-2719 had a similar biofilm mass compared with the C. albicans clinical strains (Fig. 1a). Biofilm growth activity (XTT assay) of the C. albicans laboratory reference and clinical strains varied dependent on the starting inocula, and only the C. albicans laboratory reference ATCC MYA-2719 had a similar growth activity at each starting inocula compared with the clinical isolates (Fig. 2a). Increasing starting inocula did not affect biofilm mass of the clinical strains of C. hystalae and C. dublinitiensis and clinical strain of C. glabrata and the biofilm growth activity for the clinical strains of C. dublinitiensis and C. tropicalis, whereas C. glabrata (ATCC 2001) had a
significantly higher biofilm mass at $1 \times 10^3$ than at $1 \times 10^4$ cell mL$^{-1}$ $(P < 0.001; \text{Fig. 1b})$. Candida albicans (ATCC 90234) exhibited significantly $(P < 0.001)$ more growth activity at $1 \times 10^4$ than at $1 \times 10^7$ cell mL$^{-1}$ starting inocula (Fig. 2b).

Given that optimum Candida biofilm growth in 72 h was obtained using a $1 \times 10^6$ cell mL$^{-1}$ initial yeast concentration for most strains, this concentration was therefore used for any further analysis of biofilm formation for all Candida strains.

**Comparison and categorization of the biofilm-forming ability of clinical isolates and laboratory reference C. albicans and Candida species**

Biofilm mass assessment using CV staining demonstrated that in comparison with other strains in the C. albicans group, three strains, ATCC 32354, ATCC MYA-2876 and ATCC 90234, showed the highest biofilm mass in descending order, respectively, and these had a significantly $(P < 0.05)$ higher biofilm mass than all of the other C. albicans strains (Fig. 1a). In the non-albicans Candida group, the highest biofilm mass was recorded for the clinical strain C. parapsilosis followed by C. krusei ATCC 6258, clinical isolate C. tropicalis and the reference strain C. tropicalis ATCC 750, and each had a significantly $(P < 0.05)$ higher biofilm mass than all of the other non-albicans Candida strains (Fig. 1b).

Analysis of biofilm growth activity (XTT assay) showed that the reference C. albicans strains ATCC MYA-2876, ATCC 32354, ATCC 90028 and ATCC 18804 formed the most active biofilms $(P < 0.05; \text{Fig. 2a})$. The reference non-albicans Candida strains C. parapsilosis, C. tropicalis, C. lusitaniae and C. guilliermondii had the highest biofilm growth activity, and each had significantly $(P < 0.05)$ higher growth activity than all of the other non-albicans Candida strains (Fig. 2b).

Comparing the clinical with laboratory reference C. albicans strains, ATCC strains 32354, 90234 and MYA-2876 showed significantly $(P < 0.05)$ higher biofilm mass than the clinical strains (i.e. C. albicans genotypes A–C; Fig. 1a), while in the non-albicans Candida, only the ATCC strain of C. krusei had a significantly $(P < 0.05)$ higher biofilm mass than the respective clinical strain with the remaining laboratory reference strains having less or same biofilm mass in comparison with their respective clinical isolates (Fig. 1b). Significant $(P < 0.05)$ differences were observed between the clinical and reference strains of the same species with the majority of clinical strains having less active biofilm than their reference strains (Fig. 2a and b).

When ACC and BST assays were used to compare biofilm mass of all Candida species and strains, a significant

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**Table 1.** Biofilm mass analysis data (72 h) of nine isolates of Candida as determined by ACC, BST and CV assays and CFU counts

<table>
<thead>
<tr>
<th>Candida strain</th>
<th>CFU $\log_{10} \pm SD$</th>
<th>CV absorbance $(620 \text{ nm}) \log_{10} \pm SD$</th>
<th>ACC $\log_{10} \pm SD$</th>
<th>BST absorbance $(520 \text{ nm}) \log_{10} \pm SD$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans ATCC 32354</td>
<td>11.224 ± 0.081</td>
<td>2.674 ± 0.159</td>
<td>9.279 ± 0.1</td>
<td>2.699 ± 0.167</td>
</tr>
<tr>
<td>C. albicans ATCC 90234</td>
<td>10.419 ± 0.107</td>
<td>2.318 ± 0.255</td>
<td>8.885 ± 0.077</td>
<td>1.664 ± 0.171</td>
</tr>
<tr>
<td>C. glabrata clinical</td>
<td>10.159 ± 0.138</td>
<td>0.240 ± 0.026</td>
<td>8.360 ± 0.056</td>
<td>0.329 ± 0.014</td>
</tr>
<tr>
<td>C. albicans ATCC MYA-2876</td>
<td>11.018 ± 0.224</td>
<td>1.931 ± 0.309</td>
<td>9.008 ± 0.029</td>
<td>2.128 ± 0.036</td>
</tr>
<tr>
<td>C. albicans genotype B clinical</td>
<td>9.882 ± 0.18</td>
<td>0.508 ± 0.151</td>
<td>8.655 ± 0.091</td>
<td>1.062 ± 0.151</td>
</tr>
<tr>
<td>C. albicans ATCC 18804</td>
<td>9.291 ± 0.056</td>
<td>0.139 ± 0.019</td>
<td>8.015 ± 0.083</td>
<td>0.285 ± 0.055</td>
</tr>
<tr>
<td>C. parapsilosis clinical</td>
<td>11.545 ± 0.082</td>
<td>1.720 ± 0.129</td>
<td>8.938 ± 0.119</td>
<td>1.61 ± 0.311</td>
</tr>
<tr>
<td>C. dublinensis ATCC MYA-646</td>
<td>9.730 ± 0.461</td>
<td>0.542 ± 0.108</td>
<td>8.231 ± 0.120</td>
<td>0.544 ± 0.130</td>
</tr>
<tr>
<td>C. albicans genotype A clinical</td>
<td>10.150 ± 0.779</td>
<td>0.226 ± 0.056</td>
<td>8.528 ± 0.260</td>
<td>0.906 ± 0.509</td>
</tr>
</tbody>
</table>

Data represent means and standard deviation of the above assays performed in triplicates and on two separate occasions.

**Table 2.** Correlation between assays used for biofilm mass assessment of Candida isolates

<table>
<thead>
<tr>
<th>Biofilm mass assay</th>
<th>CV</th>
<th>CFU $P &lt; 0.001$</th>
<th>ACC $r = 0.865^*$</th>
<th>BST $r = 0.903^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>1.14 ± 0.98*</td>
<td>$r = 0.726^*$</td>
<td>$r = 0.874^*$</td>
<td></td>
</tr>
<tr>
<td>CFU</td>
<td>$P &lt; 0.001$</td>
<td>10.4 ± 0.76*</td>
<td>$r = 0.874^*$</td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
<td>8.65 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>BST</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
<td>1.25 ± 0.82*</td>
<td></td>
</tr>
</tbody>
</table>

*Mean and standard deviation of biofilm mass as detected by the biofilm assays for nine Candida isolates.

†Pearson’s correlation coefficient.
(P < 0.01) positive relationship between CV and both ACC and BST methods (\( r = 0.61 \) and \( r = 0.755 \), respectively) was observed. Correlation analysis of biofilm mass (CV, ACC and BST assays) with its growth activity (XTT absorbance) revealed a weak positive correlation (\( r = 0.355, r = 0.318 \) and \( r = 0.413 \), respectively) between both biofilm variables. This allowed us to clearly differentiate the Candida isolates into different categories. Candida strains could be categorized based on biofilm mass (as measured by CV) and growth phase (as measured by XTT) of the biofilm into high, medium and low biofilm mass and actively growing and stationary. Mean and half the mean values of both CV and BST assay data were used to categorize strains into low biofilm mass/density strains (absorbance = 0–0.53, 0–0.58, respectively), medium biofilm mass/density strains (absorbance = 0.53–1.07, 0.58–1.17, respectively) and high biofilm mass/density strains (absorbance > 1.07, 1.17, respectively), while mean of XTT assay data was used to divide strains into actively growing biofilms (absorbance > 0.4) and stationary biofilms (absorbance < 0.4). Combining these categories resulted in strains being classified according to their biofilm properties (biofilm mass and growth activity) into six classes. Using CV assay for biofilm mass determination, these classes were class one (high mass and actively growing biofilms): \( \text{C. albicans} \) ATCC MYA-2876, \( \text{C. albicans} \) ATCC 32354, \( \text{C. tropicalis} \) ATCC 750; class two (high mass and stationary biofilms): \( \text{C. parapsilosis} \) ATCC 90234, \( \text{C. kruzei} \) ATCC 6258, \( \text{C. tropicalis} \) ATCC 750; class three (medium mass and actively growing biofilms): \( \text{C. albicans} \) ATCC MYA-646, \( \text{C. dubliniensis} \) ATCC 6258, \( \text{C. tropicalis} \) ATCC 750; class four (medium mass and stationary biofilms): \( \text{C. parapsilosis} \) ATCC 90234, \( \text{C. kruzei} \) ATCC 6258, \( \text{C. tropicalis} \) ATCC 750; class five (low mass and actively growing biofilms): \( \text{C. albicans} \) ATCC MYA-28719, \( \text{C. dubliniensis} \) ATCC MYA-646, \( \text{C. kruzei} \), \( \text{C. dubliniensis} \) class five (low mass and actually growing biofilms): \( \text{C. lusitaniae} \) ATCC 34449, \( \text{C. albicans} \) ATCC 18804; and lastly, class six (low mass and stationary biofilms): \( \text{C. guillermondii} \), \( \text{C. glabrata} \) ATCC 2001.

In terms of distribution of clinical and laboratory reference strains according to the above classification, all of
the \textit{C. albicans} genotypes, A–C had a medium biofilm mass and stationary (low growth rate) with no significant differences in biofilm mass and growth activity among the three genotypes. Only one \textit{C. albicans} laboratory strain matched the biofilm development of \textit{C. albicans} genotypes, which was ATCC MYA-2719. All of the other \textit{C. albicans} laboratory (ATCC) strains tested had either a higher or lower biofilm mass or growth rate. Of all non-\textit{albicans} \textit{Candida} species tested, only \textit{C. dubliniensis} and \textit{C. glabrata} laboratory and clinical isolates had similar biofilm development that being a medium biofilm mass and a low growth rate, and low biofilm mass and a low growth rate, respectively. In general, all of the \textit{C. albicans} and non-\textit{albicans} \textit{Candida} clinical isolates tested had a medium-to-low biofilm mass and a low growth rate with the exception of \textit{C. tropicalis} and \textit{C. parapsilosis}, which had a high biofilm mass, but low growth rate at 72 h.

Using BST assay for determining biofilm mass, 16 strains had the same classes as in the CV except for 6 strains that showed different distribution according to biofilm mass with \textit{C. albicans} ATCC 90028, \textit{C. lusitaniae} ATCC 34449, \textit{C. guilliermondii} ATCC 6260, \textit{C. dubliniensis} ATCC MYA-646 and \textit{C. krusei} having a higher biofilm mass and the clinical isolate \textit{C. dubliniensis} having a lower biofilm mass as determined using the BST method compared with the CV method (Fig. 3b).

Using ACC assay for determining biofilm mass, mainly two categories of biofilm mass/density could be recognized based on the mean of data: the high biofilm mass strains (log$_{10}$ cell number > 8.6) and low biofilm mass strains (log$_{10}$ cell number < 8.6; Fig. 3c). Combining XTT and ACC data resulted in strains being classified similarly to the above classification except that medium biofilm mass is not included in the ACC method (i.e. four classes; Fig. 3c). In comparison with the CV method all of the \textit{Candida} species that had a high biofilm mass as determined using CV also had a high biofilm mass as determined using the ACC method. Five strains showed a different distribution according to biofilm mass were \textit{C. albicans} ATCC 90028, \textit{C. albicans} genotype B, \textit{C. lusitaniae} ATCC 34449, \textit{C. guilliermondii} ATCC 6260 and the clinical isolate \textit{C. krusei} having a higher biofilm mass as determined using the ACC method compared with the CV method. Interestingly, the clinical isolate \textit{C. dubliniensis} had the lowest biofilm mass of all of the \textit{Candida} species.
Fig. 3. *Candida* species differentiation into distinct subgroups or classes according to their biofilm mass and growth activity. *Candida* species biofilm mass and growth activity as measured by: (a) CV absorbance vs. XTT salt reduction (XTT), respectively; (b) BST obtained by spectrophotometer (NanoDrop) vs. XTT, respectively; (c): log_{10} of cell number obtained by ACC vs. XTT, respectively. Continuous line represents the mean, while broken line represents half the mean value. 1: class one high mass and actively growing biofilms; 2: class two high mass and stationary biofilms; 3: class three medium mass and actively growing biofilms; 4: class four medium mass and stationary biofilms; 5: class five low mass and actively growing biofilms; and 6: class six low mass and stationary biofilms. Biofilm-forming ability was assessed at $1 \times 10^7$ cell mL^{-1} initial cell concentration. Data are means of experiments performed in triplicate and on two separate occasions. Filled symbols were assigned for the clinical strains, and the unfilled symbols point to the respective laboratory reference strains.

*Candida* species and strains can be identified on the graph as following: (●-A) *Candida albicans* genotype A clinical; (●-B) *C. albicans* genotype B clinical; (●-C) *C. albicans* genotype C clinical; (○-1) *C. albicans* ATCC 18804; (○-2) *C. albicans* ATCC 32354; (○-3) *C. albicans* ATCC 90028; (○-4) *C. albicans* ATCC 90234; (○-5) *C. albicans* ATCC MYA-2719; (○-6) *C. albicans* ATCC MYA-2876; (■) *Candida glabrata* ATCC 2001; (▲) *C. glabrata* clinical; (♦) *Candida parapsilosis* ATCC 22019; (◊) *C. parapsilosis* clinical; (□) *Candida krusei* ATCC 6258; (◇) *C. krusei* clinical; (►) *Candida tropicalis* ATCC 750; (◆) *C. tropicalis* clinical; (❖) *Candida guilliermondii* ATCC 6260; (❖) *C. guilliermondii* clinical; (+) *Candida dubliniensis* ATCC MYA-646; (❖) *C. dubliniensis* clinical; (+) *Candida lusitaniae* ATCC 34449.
species, which was in accordance with the biomass as determined using the BST method (Fig. 3c).

Discussion

The present study has shown that medically important Candida species and strains vary in their in vitro biofilm-forming ability. Our study is the first to evaluate multiple clinical and laboratory reference strains of C. albicans and non-albicans species of Candida. Typically research on Candida biofilms uses laboratory-adapted strains that will have been subcultured multiple times and even for decades in some cases since their first isolation, and thus, they may not be a suitable representative for biofilm formation for current clinically relevant strains (Ramage et al., 2001; Andes et al., 2004). Fungal micro-evolution has been shown to occur under in vitro and in vivo growth conditions and affect major virulence factors (Franzot et al., 1998; Fries & Casadevall, 1998). This highlights the need for adequate attention to storage conditions of both clinical and laboratory strains and the need for careful selection of an appropriate laboratory strain for biofilm studies.

Significant variation was seen in biofilm mass and growth activity between clinical and laboratory reference strains. Of interest, the majority of clinical strains showed less biofilm growth activity in comparison with reference strains. Biofilm formation is one part of the Candida virulence factors repertoire that has been shown to affect colonization, infection of the human host, as well as survival in a changing environment. These factors include adhesins, morphogenesis or the morphological transition from yeast to filamentous form (hyphae or pseudohyphae); hydrolytic enzyme production (mainly secreted aspartyl proteinases and phospholipases); and phenotypic switching (Calderone & Fonzi, 2001). Ramage et al. (2012) have shown that there was no significant difference in biofilm formation between Candida isolated from patients with denture-associated stomatitis and healthy controls; rather, significantly greater amount of secreted aspartyl proteinases was expressed from biofilms of denture-associated stomatitis isolates. Furthermore, a number of studies have shown that C. albicans and non-albicans species that form biofilms and have a low biofilm growth activity, as measured by XTT assay, are invasive and associated with disease, whereas Candida species that have a high biofilm mass and growth activity are noninvasive (Kuhn et al., 2002; Silva et al., 2010; Tobudic et al., 2011). The low biofilm growth activity seen in clinical isolates has been suggested as a mechanism for antimicrobial resistance (Mah & O’Toule, 2001; Silva et al., 2010). The presence of low bioactive cells within the biofilm may minimize the rate that antimicrobial agents are taken into the cell, thus affecting inactivation kinetics. However, metabolic quiescence is only one of multiple mechanisms that have been proposed for biofilms resistance to antimicrobials (Mah & O’Toole, 2001), and previous observations have shown that highly antimicrobial-resistant C. albicans biofilm cells were metabolically active (Kuhn et al., 2002). Seneviratne et al. (2008) have shown that biofilm formation and its architecture rather than biofilm mass correlate with Candida species virulence. Our data corroborate these findings that all of the Candida species clinical isolates had low biofilm growth activity, but varying biofilm masses. Further, it indicates that Candida species virulence cannot be ascribed solely to biofilm mass, but needs to include other measures such as biofilm growth activity. Using biofilm growth activity as a discriminatory measure, the laboratory reference strains C. albicans ATCC 90234, C. dubliniensis ATCC MYA-646 and C. krusei ATCC 6258 may have similar virulence traits compared with clinical isolates and would warrant inclusion in future studies correlating biofilm-forming ability and virulence traits, such as antifungal susceptibility or extracellular enzyme production.

We have developed straightforward, albeit biofilm structure-disruptive, cellular nondestructive methods that allow rapid biofilm mass quantification. The amount of biofilm formation assessed by both ACC and BST assays (Table 1) was found to correlate well with CFU, the gold standard method for biofilm growth assessment (Table 2). Moreover, both new assays correlated significantly with CV assay in the main biofilm study. This may indicate the applicability of the two methods as additional tools for biofilm mass estimation. Interestingly, the BST assay had a higher correlation with CV staining than the ACC method. This was reflected by the consistency in the distribution pattern of 16 strains according to their biofilm growth activity (Table 2). Moreover, both new assays correlated significantly with CV assay in the main biofilm study. This may indicate the applicability of the two methods as additional tools for biofilm mass estimation. Interestingly, the BST assay had a higher correlation with CV staining than the ACC method. This was reflected by the consistency in the distribution pattern of 16 strains according to their biofilm mass and growth activity using both methods. The categorization of Candida strains into low, medium and high biofilm mass by both CV and BST assays, in contrast to ACC assay, may indicate that the former assays are more discriminative than the ACC method. Additionally, problems of cell number underestimation by ACC compared with the gold standard CFU counting were observed (Table 1). In contrast to the CV method, with the BST method, it is feasible to reseed the biofilm isolated cells, after measuring its mass, to test the effect of different antibiofilm applications and to assess the level of enzymes or genes expressed in the same biofilm.

In this study, we also evaluated the effect of initial cell inocula on biofilm formation to optimize the cell concentration required for biofilm formation within a 72-h assay. Our results indicate that although it was possible to get confluent biofilms for some Candida strains at low cell concentrations, biofilm formation of a majority of
yeast increased with the increase in amount of initial candidal cell inocula. On the other hand, in a paradoxical manner, some species or strains produced biofilm whose mass (e.g. reference strain C. lusitaniae) or growth activity (e.g. clinical strain C. dubliniensis) was not affected by the amount of cells present and even significantly higher mass (reference strain C. glabrata) or growth activity (reference strain C. albicans ATCC 90234) were observed at low initial cell concentration. This may be attributed to the different yeasts metabolic requirements for biofilm development or perhaps the high nutritional requirements as a consequence of increased cellular density, affecting that individual strain’s ability to form biofilms. Further research involving large number of strains is required to examine the effect of amount of the environmental dietary content on biofilm growth.

The XTT reduction assay used to assess biofilm metabolic activity has previously been shown to be a reliable semi-quantitative method for estimation of biofilm growth (Ramage et al., 2001; Tumbarello et al., 2007). However, the measured biofilm activity was not always associated linearly with the biofilm cellular mass most likely due to cells in the biofilm having different metabolic activities. The biofilm cells are enclosed in a case of exopolymeric matrix that may limit the homogenous diffusion of nutrients and oxygen, resulting in possible alteration in the cellular metabolic activity (Henriques et al., 2006). Another possible explanation for this weak association between biofilm mass and activity could be that some of the tested yeasts have reached maximum biofilm mass and stopped growing at the time of assay (stationary growth state); reflected by the lower activity as detected by the XTT reduction method. Although previous data indicate that XTT assay is useful for comparisons of biofilm biomass within one strain at different time intervals (Kuhn et al., 2003), it may be problematic for interstrain comparisons as the ability of different isolates to metabolize XTT is not constant (Kuhn et al., 2003). Thus, in the present study, the XTT assay was used for interstrain comparisons in biofilm growth activity as previously reported (Silva et al., 2010).

The observations of both CV and XTT assays have confirmed that an individual strain of oral yeast has unique biofilm mass and activity. This resulted in the tested yeasts being differentiated into distinct categories regarding their biofilm-forming ability. All laboratory strains formed biofilms of differing categorization compared with the clinical isolates except three laboratory Candida strain (ATCC C. albicans MYA-2719, ATCC strains of C. dubliniensis and C. glabrata) shared the same biofilm properties of their respective clinical strains.

We believe that the current study is novel in providing information about biofilm growth behaviour (using different initial cell inocula) and variations in biofilm-forming ability among all medically important Candida species. This is the first investigation that places an emphasis on the variations between clinical and laboratory reference strains of the same Candida species. Such differences between clinical and laboratory reference strains need to be taken into consideration when Candida biofilm researchers use reference strains to draw important clinical conclusions. Furthermore, the current study presents very straightforward methodology that assesses biofilm mass without compromising biofilm cell structure, although it disrupts biofilm structure. This is important when there is a need to immediately analyze virulence factors in single biofilm cells using techniques such as flow cytometry and cytometry by time-of-flight mass spectrometry and to reseed biofilm cells after biofilm mass assessment to do further analysis on fungal biofilms.

In conclusion, this study highlights that C. albicans and Candida species laboratory reference strains and clinical isolates form biofilms at different rates and that the selection of a laboratory reference strain for biofilm studies needs to take into account the biofilm-forming ability of the clinically relevant species.

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References