Comparing apples and oranges: considerations for quantifying candidal biofilms with XTT and the need for standardized testing

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Editorial

For laboratories with limited resources the economical study of *Candida* spp. biofilms tends to lend itself to unsophisticated models that negate the requirement for expert handling or the use of specialized equipment. Within these constraints, models that are readily amenable to high throughput screening are highly desirable and widely utilised. One key standardized high throughput model that has been extensively detailed is a 96 well microtitre plate format with flexibility to study the formation of biofilms and their antifungal susceptibilities (Ramage *et al.* 2001; Pierce *et al.* 2008). This has subsequently been adopted by a number of groups to evaluate various experimental parameters of biofilm formation (Ramage, Vande Walle et al. 2001; Thein *et al.* 2007; Tumbarello *et al.* 2007). Alongside this biofilm testing platform is a simple soluble formazan based bioassay that uses the metabolic dye XTT (2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) (Tellier *et al.* 1992; Hawser 1996a), enabling a rapid and highly reproducible semi-quantitative assessment of biofilms (Hawser 1996b; Hawser *et al.* 1998; Ramage, Vande Walle et al. 2001). This colorimetric assay is non-invasive and non-destructive, requiring minimal post-processing of samples as compared to other alternative methods, such as viable cell counts that run into problems with cellular aggregates. Using this technique multiple microtitre plates can be processed simultaneously without compromising accuracy. However, whereas the XTT assay is useful for antifungal testing to evaluate the effects of the drug on a sessile population, in comparison to an untreated control, metabolic variability between different isolates and species, making its usefulness in quantifying biofilm development limited. Therefore, caution should be taken when interpreting the data obtained from this metabolic assay to assess biofilm formation (Kuhn *et al.* 2003; Taff *et al.* 2012).

Recently there is an increasing wave of candidal biofilm research using a rapid screen of isolates with crystal violet (CV) based biomass assays and the metabolic dye XTT (Dhale *et al.* 2014; Marcos-Zambrano *et al.* 2014). These are used as a means of comparing multiple clinical isolates and species, particularly in relation to clinical outcomes (Tumbarello, Posteraro et al. 2007; Tumbarello *et al.* 2012; Rajendran *et al.* 2016). The recent study by Pongrácz and colleagues (2016) is worth considering in this context. In the paper entitled "*in vitro* biofilm production of *Candida* bloodstream isolates: any association with clinical characteristics?" (Pongrácz *et al.* 2016). The authors used both CV and XTT to classify biofilm producers. However, there is no apparent criteria or basis for stratification. For example, the authors used OD_{490} for standard XTT concentrations, where values between 0.09 and 0.45 to

denote low biofilm formers (LBF) and values ≥ 0.9 to denote high biofilm formers (HBF). Whereas, for CV an OD_{570} of ≥ 0.09 were simply considered biofilm producers. In contrast, Tumbranello and colleagues (2007) used both standard XTT methodology accompanied by spectrophotometric analysis (% transmittance), which was stratified using an ordinal scale (Tumbarello, Posteraro et al. 2007). Stratification was used to group non-biofilm formers, LBF and HBF, and to correlate with XTT readings. This group later used the same methodology, though stating that % transmittance of <10 equated to non-biofilm formers, and for XTT anything above an OD_{490} of 0.1 was a biofilm former (Tumbarello, Fiori et al. 2012). Finally, our own group used a similar approach to categorize isolates based three bioassays, XTT, SYTO 9 and CV biomass (OD_{570}) values (Rajendran, Sherry et al. 2016). CV was finally used to stratify the clinical isolates tested, and those within the first quartile (Q1) were classed as LBF, isolates with a biomass greater than the third quartile (Q3) were classed as HBF, and those in between were classed as intermediate biofilm formers (IBF) (second quartile [Q2]). Clearly, a variety of different criteria are used in these published studies, but should we be guided by just one criterion? This is important if we are going to try as a community to correlate the clinical importance of candidal biofilm infections.

For the novice entering the world of candidal biofilm research then these, or even the experienced, the plethora of papers taking differing approaches can be confusing,. Which of these quantitative methods is the most robust and reliable? Should I be comparing different species using these methods? Is there a defined number that differentiates biofilm from non-biofilm? Are there different levels of biofilm formation? Lack of clarity and standardization in the field makes answering these questions impossible, particularly as there are other permutations to consider such as choice of media, time of biofilm development, the specifics of their own quantification method, and of course the purpose of the experiment. Clearly, we do need some guidance that will allow those using these assays to undertake meaningful comparisons with the published literature. Having had the opportunity to publish and review in this field over the past 15 years or so then there are aspects that should be adhered to: 1) do not use XTT to compare different species due to variability in XTT readings (you are comparing apples and oranges), 2) understand the limitations of CV (the assay is not sensitive enough to differentiate subtle differences in biofilm formation, 3) when screening clinical isolates or any panel of isolates, take a belt and braces approach (one bioassay is not enough) and 4) understand your research question (the bioassay(s) you select to use is dictated by this).

There are many other do's and don'ts when it comes to investigating candidal biofilms, but the one key pointer is to take a balanced approach and read both the contemporary and historical literature. Both are equally valuable, as we can see from the evolution of the subject area, but the early studies lay a solid foundation. One aspect remains constant however, the use of XTT as our primary tool. Remember its limitations though, and use the quantitative data produced in a meaningful way in order to detect important clinical correlations, that may be missed otherwise. Pongrácz and colleagues (2016) suggest from their analysis that biofilm formation is greater in non-albicans yeasts than *C. albicans*, and that biofilm production does not correlate with mortality (Pongrácz, Benedek et al. 2016), which is in contrast to similar recent study designs (Tumbarello, Fiori et al. 2012; Rajendran, Sherry et al. 2016). We therefore need to ensure that in addition to having robust clinical data that due consideration is given to the accompanying technical analysis in defining the parameters of what really constitutes a *Candida* biofilm.

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