Comparing apples and oranges: considerations for quantifying candidal biofilms with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] and the need for standardized testing

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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 utilized. 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 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 semiquantitative assessment of biofilms (Hawser, 1996b, 1998; Ramage et al., 2001). This colorimetric assay is non-invasive and non-destructive, requiring minimal post-processing of samples as compared with 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, the XTT assay is only useful for direct comparisons, e.g. antifungal testing to evaluate the effects of the drug on a sessile population in comparison with an untreated control. Metabolic variability between different isolates and species makes 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 has been an increasing wave of candidal biofilm research using a rapid screen of isolates with crystal violet-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 et al., 2007, 2012; Rajendran et al., 2016). The recent study by Pongrácz et al. (2015) 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., 2015), the authors used both crystal violet and XTT to classify biofilm producers. However, there was no apparent criterion or basis for stratification. For example, the authors used OD_{490} for standard XTT concentrations, where values between 0.09 and 0.45 denoted low biofilm formers and values ≥0.9 denoted high biofilm formers. Whereas for crystal violet an OD_{570} ≥0.09 was simply considered to denote biofilm producers. In contrast, Tumbarello et al. (2007) used standard XTT methodology accompanied by spectrophotometric analysis (percentage transmittance), which was stratified using an ordinal scale. Stratification was used to group non-biofilm formers, low biofilm formers and high biofilm formers, and to correlate with XTT readings. This group later used the same methodology, though stating that a percentage transmittance of <10 equated to non-biofilm formers and for XTT anything above OD_{490} 0.1 was a biofilm former (Tumbarello et al., 2012). Finally, our own group used a similar approach to categorize isolates based on three bioassays: XTT, SYTO 9 and crystal violet biomass (OD_{570}) values (Rajendran et al., 2016). Crystal violet was finally used to stratify the clinical isolates tested, and those within the first quartile were classed as low biofilm formers, isolates with a biomass greater than the third quartile were classed as high biofilm formers and those in between were classed as intermediate biofilm formers (second quartile). Clearly, a variety of different criteria are used in these published studies, but should we be guided by just one criterion? This is

**Abbreviation:** XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide.
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, 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 general rules 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 crystal violet (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, which may be missed otherwise. Pongrácz et al. (2015) suggest from their analysis that biofilm formation is greater in non-\textit{albicans} yeasts than in \textit{C. albicans} and that biofilm production does not correlate with mortality, which is in contrast to similar recent study designs (Tumbarello et al., 2012; Rajendran et al., 2016). We therefore need to ensure that in addition to having robust clinical data, due consideration is given to the accompanying technical analysis in defining the parameters of what really constitutes a \textit{Candida} biofilm.

REFERENCES


