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1 **A microtiter plate-based quantitative method to monitor the growth rate of**

2 **dermatophytes and test antifungal activity**

3

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9

10 **Abstract**

11 Dermatophytosis is one of the most common superficial fungal infections, which is mainly
12 caused by filamentous fungi such as *Trichophyton* species. A challenging aspect in
13 dermatophyte research is the lack of a straightforward method to measure the rate of growth,
14 in particular when growing dermatophytes in small volumes such as in microtitre plates.
15 However, one characteristic of dermatophytes is their ability to produce compounds such as
16 ammonia that make the growth medium more alkaline. The objective of this study was to test
17 whether the change in pH in a liquid medium, colourimetrically established using the indicator
18 phenol red, was linearly and directly proportional to the growth rate for *Trichophyton rubrum*
19 and *Trichophyton interdigitale*. The changes in the colour determined by the phenol-red based
20 assay showed a good correlation with the amount of fungal biomass over an incubation period
21 of 24-120 hours. The functionality of the phenol red assay was also validated in experiments
22 on the growth of *T. rubrum* in the presence of antifungals. The changes in colour showed a
23 clear dose-response relationship compounds and enabled determination of the minimum
24 inhibitory concentration. The phenol red assay is thus a simple and straightforward assay to
25 monitor the rate of growth of *Trichophyton* spp. and test antifungal activity.

26

1. Introduction

Dermatophytosis is one of the most common fungal infections of keratinised tissues such as nails, hair and skin, with about 20 to 25% of the world population being infected (Havlickova et al., 2008). They are most commonly caused by dermatophytes, which are fungi that can degrade keratin. *Trichophyton rubrum* and *Trichophyton interdigitale* are the two most common causative agents, causing over 90% of dermatophyte infections in 2005 in the UK (Borman et al., 2007).

With bacteria, growth in liquid cultures can be monitored easily using, for instance, the optical density (OD) of cultures. This technique does not work well for moulds due to their filamentous and heterogeneous morphology in liquid cultures. For such fungi, one could measure radial growth on agar plates, and for liquid medium, a traditional method would be determining the mycelium dry weight. However, the latter method is time-consuming due to the relatively slow growth rate of most filamentous fungi. Furthermore, it is also challenging to implement in the case of multi-replicate assays and when growing fungi in small volumes such as in microtitre plates (Arima and Uozumi, 1967; Granade et al., 1985; Matcham et al., 1984; Taniwaki et al., 2006). An alternative to dry weight measurements is by staining the fungal biomass with a dye such as crystal violet, and then measure the absorbance of the dye, but this is still fairly laborious and involves several steps (Costa-Orlandi et al., 2014).

An alternative approach to measuring fungal biomass is to use colourimetric assays that monitor metabolic activity, such as those based on resazurin or the tetrazolium salts 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and these have been applied to, for instance, antifungal susceptibility testing with *Aspergillus* species (Jahn et al., 1996; Meletiadis et al., 2002; Monteiro et al., 2012). However, dermatophytes prefer a mildly acidic environment for the initial stages of infection, as a number of important virulence factors such

52 as proteases have optimal activity at the pH that matches that of the skin (pH 5.5) and nail (pH
53 5.2) (Martinez-Rossi et al., 2012; Matousek and Campbell, 2002; Murdan et al., 2016). During
54 growth of dermatophytes, degradation of keratin or other proteins leads to release of ammonia,
55 resulting in a shift towards an alkaline pH (Mercer and Stewart, 2019). The initial low pH
56 reduces the adequacy of the MTT and XTT cell viability assays that require a neutral pH (pH
57 7.4). Indeed, it was shown that the acidic environment shifts the absorbance and reduces the
58 activity of formazan-producing enzymes (Grela et al., 2018; Johno et al., 2010; Plumb et al.,
59 1989). Moreover, the resazurin assay monitors not only cell viability but also functions as a pH
60 indicator (Lancaster and Fields, 1996), and false negative results may therefore result from the
61 shift towards alkaline pH. Therefore, better and easier assays to monitor growth and viability
62 of dermatophytes are desirable and would be useful to study e.g. antifungal susceptibility.
63 The ability of dermatophytes to producing ammonia has been exploited to develop a
64 simple and rapid dermatophyte test medium (DTM) to diagnose the presence of
65 dermatophytosis by incorporating the pH indicator phenol red, which changes colour from
66 yellow to red under alkaline conditions (Taplin et al., 1969). This colour change is
67 characteristic for dermatophytes such as *T. rubrum* and *T. interdigitale* and aids in their
68 identification It should be noted, however, that some non-dermatophytes may also cause a
69 colour change in DTM, leading to the introduction of an improved Dermatophyte Identification
70 Medium (Salkin et al., 1997; Mesquita et al., 2016; Gromadzki et al., 2003). Here we developed
71 a simple method that uses phenol red to measure the rate of fungal growth in liquid culture
72 media, and also demonstrate its usefulness to study antifungal susceptibility of dermatophytes.
73 The phenol red assay was validated by comparison with quantification of the biomass, as
74 determined using crystal violet (Costa-Orlandi et al., 2014). Compared with other fungal
75 growth and viability assays, this assay is inexpensive and straightforward, with minimal post-
76 processing that avoids artefacts.
77

78 ## 2. Materials and methods

79 *2.1 Strains and culture conditions*

80 *Trichophyton* species used in this study were *T. interdigitale* (ATCC 9533) and *T. rubrum*
81 (ATCC 28188), obtained from Fisher Scientific (Loughborough, UK). To isolate microconidia,
82 strains were cultured on potato dextrose agar (Sigma-Aldrich, St. Louis, MO, USA) for 15 days
83 at 30°C to induce full sporulation. Conidia were harvested with sterile 1% Tween-20 (Fisher
84 Scientific), filtered through a sterile cell strainer (40 µm) and resuspended in sterile Milli-Q
85 water. The conidia were aliquoted and stored at -20°C and aliquots were used within two
86 weeks, during which no significant reduction in viability was observed.

87

88 *2.2 Fungal growth assay*

89 The fungal growth assay was performed by dispensing 200 µL of Sabouraud dextrose broth
90 (SDB) with phenol red (0.002%) in 96-well flat-bottom microtitration plates (Costar, Corning,
91 N.Y., USA) and adding microconidia to each well with a final concentration of 1×10^6 CFU
92 ml^{-1}. The plate was then sealed with parafilm and incubated for 24-120 hrs on a microplate
93 shaker (150 rpm) at 30°C. Finally, the liquid from each well was transferred into a new
94 microtitration plate and then read in UV/vis multiplate reader (CLARIOstar Plus, BMG
95 Labtech, Aylesbury, UK) at a wavelength of 560 nm.

96

97 *2.3 Quantification of biomass by crystal violet staining*

98 *Trichophyton* spp. biomass quantification was done by crystal violet staining, as described by
99 Costa-Orlandi et al. (2014), with some slight modifications. Briefly, after growth of the fungi
100 in 96-well plates in SDB, the solution from each well was removed, the fungal biomass at the
101 bottom of each well was washed twice with PBS, and then the plate was dried in an oven for
102 20 min at 60°C. The biomass in each well was then stained for 5 min with 150 µL of 0.1%

103 crystal violet solution. The solution was carefully removed, and wells were submerged three
104 times in trays with cold tap water to remove excess stain. After that, 200 μL of 95% ethanol
105 was added to each well to dissolve the crystal violet. After mixing thoroughly, the solution was
106 transferred to a new microtitration plate and read in a UV/Vis multiplate reader at a wavelength
107 of 595 nm.
108
109 *2.4 Antifungal susceptibility test*
110 The potential of an antifungal susceptibility test based on the phenol red assay was tested with
111 nystatin and EDTA. 200 μL of SDB with phenol red (0.002%) and different concentrations of
112 EDTA or nystatin (0.1-256 μg/mL) was dispensed in 96-well plates, followed by adding *T.*
113 *rubrum* conidia to each well at a final concentration of 1×10^6 CFU ml^{-1}. The plate was
114 incubated for 72 hours on a microplate shaker (150 rpm) at 30°C, after which the solutions
115 from each well were transferred to a new plate and the absorbance at a wavelength of 560 nm
116 was determined using a multiplate UV/vis spectrometer.
117 To apply the phenol red assay by addition after growth, cells were grown as above in
118 SDB (without phenol red) in the presence of antifungal compounds (nystatin, cycloheximide,
119 terbinafine hydrochloride or clotrimazole). After 72 hours the supernatant was transferred to a
120 new plate containing 2 μl 0.2% phenol red (in DMSO) in each well.
121
122 *2.5 Calculation and statistical analysis*
123 The results were presented as the mean ± standard deviation. Since the response of absorbance
124 is different in each assay, the results from the phenol red and crystal violet biomass assays were
125 expressed using the following equation (Eq.1) and plotted against incubation times.
126

$$\%\ \text{percentage change} = \frac{(\text{Sample at 120 hrs}_{\text{Abs}} - \text{Blank}_{\text{Abs}})}{(\text{Sample}_{\text{Abs}} - \text{Blank}_{\text{Abs}})} \times 100\% \qquad (Eq.1)$$

127 The slope and intercept of these linear regressions were compared by analysis of covariance
128 (ANCOVA), performed by GraphPad Prism software. Statistical significance was considered
129 for *p*-values less than 0.05.

130 The growth inhibition rate of antifungal susceptibility test was calculated by the
131 equation below (Eq.2), and the data were plotted against concentrations of the antifungal
132 agents. The minimum inhibitory concentration (MIC) is defined as the lowest concentration
133 that gives at least 90% growth inhibition as measured using the phenol red assay.

134 $$\%\ \text{growth inhibition} = \left[1 - \frac{(\text{Sample}_{\text{A560}} - \text{Blank}_{\text{A560}})}{\text{Control}_{\text{A560}}}\right] \times 100\% \quad (Eq.2)$$

135 3. **Results**

136 *3.1 Correlation between the phenol red growth assay and biomass quantification*

137 The pH of *Trichophyton* cultures grown in Sabouraud broth shifted from 5.5 to 8.0 throughout
138 120 hrs (data not shown). The indicator phenol red has an absorption peak at 430 nm at low
139 pH, whereas the absorption maximum shifts to 560 nm between pH 6.8-8.2, which results in
140 the change from yellow to red in the culture medium. We examined whether this colour change
141 could be used to measure the growth of *T. rubrum* and *T. interdigitale* by following the
142 absorbance of the culture supernatant containing phenol red at 560 nm in the period 24-120
143 hours after inoculation. Incubation times shorter than 24 hours showed no change in the colour
144 of phenol red, while with longer periods of time there was so much biomass that it became
145 difficult to extract the liquid from the wells. The results from the phenol red assay were
146 compared with the data on biomass formed as determined using crystal violet staining. As
147 shown in Fig 1A and 1B, the two assays show very similar results, with no significant
148 difference found between the slopes or intercepts of the two linear regressions as determined
149 by ANCOVA. Very similar results were also obtained when comparing the phenol red assay
150 with release of protein and DNA after treatment in lysis buffer (data not shown). However,
151 complete lysis was difficult to achieve; as it was unclear whether the level of lysis was similar
152 in all stages of growth, these results were less conclusive.

153

154 *3.2 Application of phenol red assay to antifungal susceptibility testing*

155 To further test the usefulness of the phenol red assay, the growth of *T. rubrum* was determined
156 in the presence of antifungal compounds. Two known growth inhibitors, the antifungal nystatin
157 and the chelator EDTA, were used to evaluate the susceptibility test. As shown in Fig 2, the
158 growth inhibition effect was reflected in the results of the assay, and a clear dose-response
159 relationship between the growth and the concentration of inhibitors was observed. The curves

in Fig. 2 were used to determine the MIC for nystatin and EDTA, which were 3 µg/mL and 48 µg/mL, respectively.

We also tested whether the susceptibility testing can be done by adding a stock solution of phenol red after fungal growth. As shown in Figure 3, we could visualise inhibition of *T. rubrum* growth in the presence of terbinafine (inhibition at <0.01 µg/mL), clotrimazole (<0.1 µg/mL and nystatin (<10 µg/mL), whereas no inhibition was observed with cycloheximide. The latter antifungal is used in DTM as it inhibits saprophytic fungi whereas dermatophytes are resistant to cycloheximide.

170 ## 4. Discussion

171 Given their relevance to human health, improved techniques to study the antifungal drug
172 therapies are required. Due to the heterologous morphology of filamentous fungi, the typical
173 measurements of microbial growth and viability such as optical density and colony counting
174 cannot be used. Instead, mycelium dry weight is the most common method to measure the
175 filamentous fungal growth. However, this is time-consuming, and it is difficult to process
176 multi-replicate assays, in particular when working with small volumes (Arima and Uozumi,
177 1967; Granade et al., 1985; Matcham et al., 1984; Taniwaki et al., 2006).

178 Phenol red is a commonly used pH indicator to monitor the pH of the cell culture media,
179 based on a gradual transition in colour following pH shifts from acidic to alkaline, and *vice*
180 *versa*. Based on this, DTM (containing phenol red) was developed to differentiate
181 dermatophytes from other fungi as the only the former cause an alkaline shift on the pH of the
182 medium to 8-9, by deaminating amino acids to form ammonia as a by-product (Kunert, 2000;
183 Monod, 2008; Taplin et al., 1969). In this study, we expand the usage of phenol red so it can
184 be used as an assay to measure fungal growth and to determine antifungal activity in liquid
185 cultures. The phenol red growth assay was validated by measuring the fungal growth of *T.*
186 *rubrum* and *T. interdigitale* in SDB based on the biomass between 24-120 hours of growth.
187 Thus, alkalinisation of the medium as a result of utilising nitrogen sources is directly correlated
188 to fungal growth, demonstrating that the phenol red assay is a reliable and easy method to
189 measure growth of *Trichophyton* spp.. It should be noted that some non-dermatophytic fungi
190 such as *Hisptoplasma capsulatum* and *Blastomyces dermatitidis* also cause a similar change of
191 colour in media containing phenol red (Salkin, 1973). It is thus conceivable that the method
192 developed here may be employed for a number of these fungi, but we have not tested this.

193 The phenol red growth assay can overcome the disadvantages of various methods used
194 to measure fungal growth. Apart from traditional methods such as dry weight measurements,

195 crystal violet staining has been used to study biofilm formation of *T. rubrum* (Costa-Orlandi et
196 al., 2014) and the MIC of various antifungal compounds on filamentous biomass (Kvasničková
197 et al., 2016; Mowat et al., 2007). However, the crystal violet staining requires fungi to adhere
198 to the surface of the culture plates, and the biomass of some filamentous fungi may be removed
199 easily during the washing steps. The phenol red growth assay involves a simple procedure and
200 does not require any washing steps. We also showed that a phenol red stock solution can be
201 added after growth and can be used to visualise inhibition of several antifungal agents. The
202 values obtained corroborated earlier studies that determined the MIC for these antifungal
203 agents (Gupta and Kohli, 2003, Shadomy, 1971, Adimi et al., 2013, Agbulu et al 2015).
204 The antifungal susceptibility using phenol red was demonstrated by studying the
205 inhibitory effect of nystatin, a polyene antifungal agent, and EDTA, a chelating agent and
206 metalloprotease inhibitor to *T. rubrum* (Sen, 1964). Notably, the MIC for nystatin was the same
207 as found in a study that determined this using SDB slant cultures (Agbulu et al., 2015). The
208 sigmoidal dose-response curves were established after 72 hours incubation, a time point is
209 commonly used in studying the fungal growth of dermatophytes, and provides an optimum
210 response of the susceptibility (Costa-Orlandi et al., 2014; Smijs et al., 2008). Furthermore, at
211 72 hours it was still easy to extract liquid from the wells, which became more difficult with
212 longer incubation times. Thus, the phenol red assay provides an alternative method to
213 determine MIC values. The method does not replace existing standardised methods to
214 determine antifungal activity of filamentous fungi (Johnson, 2008), as the phenol red assay is
215 limited to dermatophytes only, but it is nevertheless a useful addition because of its ease and
216 simplicity. In conclusion, the phenol red growth assay provides a reproducible and cheap
217 method that simplifies the study of dermatophytes and enables antifungal susceptibility testing.
218

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321

322 **Fig 1.** Measurement of fungal growth of *T. rubrum* **(A)** and *T. interdigitale* **(B)** in SDB between
323 24-120 hours *via* crystal violet biomass staining. Each assay was completed with the phenol
324 red viability assay, and their rate of the percentage change versus the incubation times are did
325 not show a significant difference (ANCOVA – A-B, *ns*, $n = 3$).

326

327 **Fig 2.** Dose-response curves illustrating the antifungal susceptibility activities of nystatin **(A)**
328 and EDTA **(B)** against *T. rubrum* in SDB using the phenol red growth assay after 72 hours of
329 incubation (A: $R^2 = 0.9955$; B: $R^2 = 0.9731$; $n = 3$).

330

331 **Fig 3**. Antifungal susceptibility testing by adding phenol red after growth. The antifungals
332 used were terbinafine (Te), clotrimazole (Cl); cycloheximide (Cy), and nystatin (Ny), with
333 the concentrations used indicated above the panels.

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345 Figure 1

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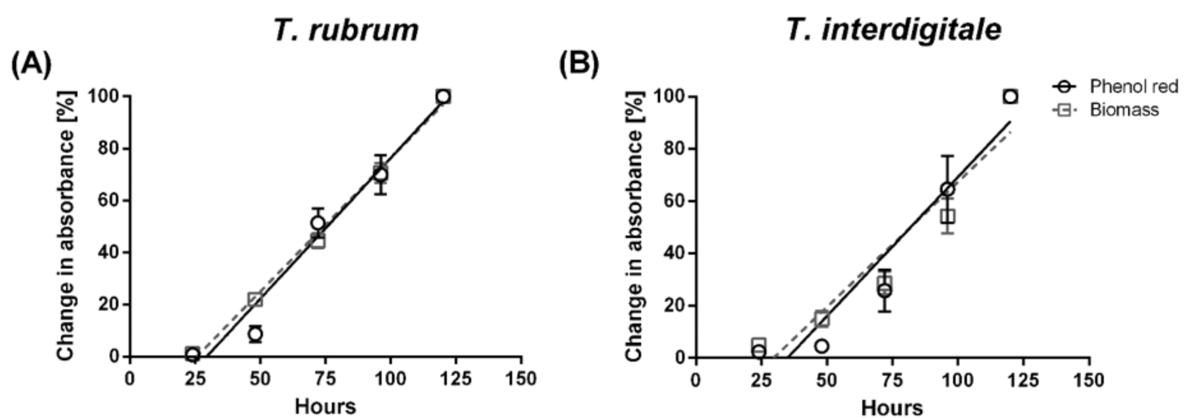

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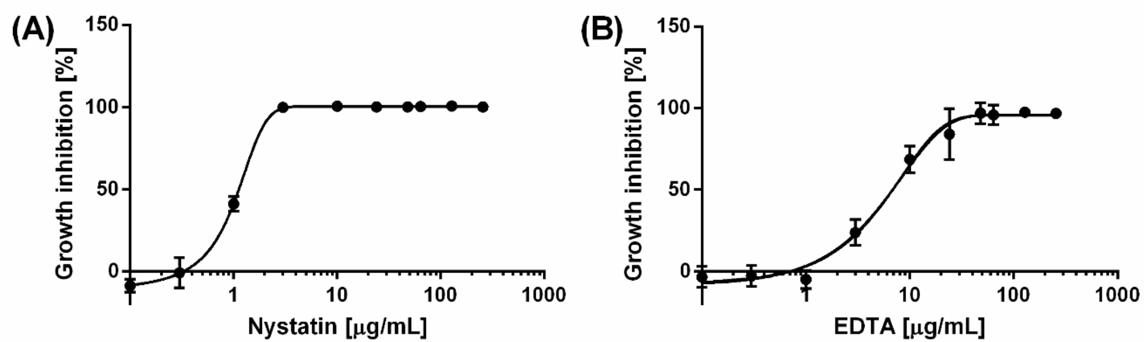
350 Figure 2



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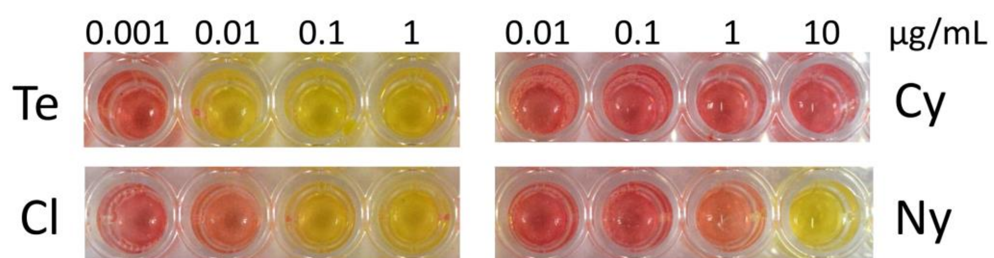
352 Figure 3



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