Viable-quantitative PCR for assessing the response of *Candida albicans* to antifungal treatment

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Abstract

Propidium monoazide (PMA) or ethidium bromide monoazide (EMA) treatment has been used before nucleic acid detection methods, such as PCR, to distinguish between live and dead cells using membrane integrity as viability criterion. The performance of these DNA intercalating dyes was compared in many studies utilizing different microorganisms. These studies demonstrated that EMA and PMA differ in their abilities to identify non-viable cells from mixed cell populations, depending on the microorganism and the nature of the sample. Due to this heterogeneity, both dyes were used in the present study to specifically distinguish dead from live *Candida albicans* cells using viable-qPCR. The viable-qPCR was optimized and the best results were obtained when pre-treating the cells for 10 min in the dark with 25 μM EMA followed by continuous photoactivation for 15 min. The suitability of this technique to distinguish clotrimazole- and fluconazole-treated *C. albicans* cells from untreated cells was then assessed. Furthermore, the antifungal properties of two commercial essential oils (*Thymus vulgaris* and *Matricaria chamomilla*) were evaluated. The viable-qPCR method was determined to be a feasible technique for assessing the viability of *C. albicans* after drug treatment and may help to provide a rapid diagnostic and susceptibility testing method for fungal infections, especially for patients treated with antifungal therapies.

Introduction

Invasive fungal infections are an increasingly recognized cause of morbidity and mortality, especially in immunocompromised patients (Marr 2009; Miceli et al. 2011), in whom, infections due to *Candida* species are the most common (Pfaller et al. 2007; Sims et al. 2005). *Candida* species produce infections ranging from non-life-threatening mucocutaneous illnesses to invasive processes that may involve disseminated disease and sepsis. Due to the severity of such infections, a rapid and effective diagnostic test is necessary to allow a prompt and effective therapeutic intervention. In addition, a fast method to test for susceptibility is desired (Pappas et al. 2004).

Identification of bacterial pathogens by traditional methods is still a crucial element of the diagnostic process. However, methods such as culturing and sub-culturing organisms, especially those that are fastidious, can be laborious and time-consuming, and may prolong a patient's definitive diagnosis and treatment. While there has been improvement in traditional methods, clinical laboratories have begun to adopt nucleic acid-based methods to identify pathogens rapidly and reliably.

Many efforts have been focused on the detection of *Candida albicans* using molecular techniques, such as quantitative PCR (qPCR) (Fricke et al. 2010; Khan et al. 2009; Maaroufi et al. 2003). However, the major disadvantage of qPCR is its inability to differentiate between viable and non-viable cells. This is especially important when attempting to diagnose and monitor disease; thus, there is an urgent need for adding viability information to DNA-based diagnostics in clinical microbiology. The DNA of dead cells is expected to remain stable over prolonged periods of time (Josephson et al. 1993); thus, DNA techniques may include the dead population and overestimate the microorganisms present in a sample. To overcome this, selective nucleic acid intercalating dyes, including ethidium monoazide (EMA) and propidium monoazide

(PMA), have been successfully used in conjunction with qPCR (herein called viable-qPCR) for a large spectrum of microorganisms: bacteria (Agustí et al. 2010; Chang et al. 2009; Elizaquível et al. 2012; Kralik et al. 2010; Kramer et al. 2009; Nocker and Camper 2006; Nocker et al. 2006; Rawsthorne et al. 2009; Takahashi et al. 2011), fungi (Andorrà et al. 2010; Rawsthorne and Phister 2009; Vesper et al. 2008), protozoans (Brescia et al. 2009; Fittipaldi et al. 2011; Thomas et al. 2012), and viruses (Fittipaldi et al. 2010; Parshionikar et al. 2010; Sánchez et al. 2012).

The viable-PCR method uses membrane integrity as the criterion to differentiate between live and dead cells. Theoretically, selective nucleic acid intercalating dyes should only penetrate into membrane compromised cells or dead cells. The presence of an azide group is believed to permit cross-linking of the dye to the DNA after exposure to strong visible light. Photolysis of EMA and PMA converts the azide group into a highly reactive nitrene radical, which can react with any organic molecule in its proximity, including DNA. In this bound state, DNA cannot be amplified by PCR (Nocker and Camper 2009; Rudi et al. 2005). This promising analytical approach is still in development and needs to be investigated further (Fittipaldi et al. 2012).

As mentioned above, different studies have shown the feasibility of viable-qPCR procedures; nevertheless, there are only four studies in fungi, which are not related with clinical applications (Andorrà et al. 2010; Rawsthorne and Phister 2009; Shi et al. 2012; Vesper et al. 2008).

In this work, for the first time, a viable-qPCR method for *C. albicans* has been optimized. Additionally, the potential of this method to evaluate diagnostic and antifungal treatments was demonstrated for four antifungal substances (clotrimazole, fluconazole, and the essential oils, *Thymus vulgaris* and *Matricaria chamomilla*).

Methods

Yeast strain and culture conditions

The *C. albicans* ATCC 2091 strain was plated on Sabouraud glucose-agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 48 h. *C. albicans* cells were harvested from the agar plates and suspended in 10 ml phosphate-buffered saline (PBS 1X, pH 7.4) to obtain a working yeast suspension. The cell density was adjusted to an OD_{600} of 0.8, corresponding to 5.0 x 10^6 cfu/ml.

Antifungal products

Clotrimazole (Sigma Aldrich, Madrid, Spain), fluconazole (Laboratorio STADA, Barcelona, Spain), and *M. chamomilla* and *T. vulgaris* essential oils (Plantis, Barcelona, Spain) were used as inactivating agents. The antifungal products were dissolved in 20% dimethyl sulfoxide (DMSO) solution (Sigma Aldrich, Madrid, Spain) to a final concentration of 10 mg/ml. The essential oils were diluted 10-fold in sterile distilled water.

Heat treatment

To obtain dead cells for optimization of the viable-qPCR procedure, 500 µl of the working yeast suspension was heated at 85 ºC for 30 min using a standard laboratory heat block. The loss of viability of the cells was tested by plating 100 µl of cell suspension on Sabouraud glucose-agar plates, followed by incubation at 37 ºC for 48 h.

Optimization of the viable-qPCR procedure

The efficiency of 50 μM EMA or PMA treatment was analyzed to choice the working intercalating dye. Once the intercalating dye was chosen, different parameters, such as dye concentration, photoactivation conditions, incubation time, and incubation temperature were evaluated to optimize the viable-qPCR method and to maximize the difference in crossing point (Cp) values between live and dead *C. albicans* cells.

EMA and PMA (Biotium, Hayward, CA, USA) were resuspended in PCR grade water (VWR International Eurolab, Barcelona, Spain) to obtain stock dye solutions of 2.97 mM and 2 mM, respectively. These stock solutions were aliquoted into dark tubes and stored at -20 ºC until needed.

The EMA and PMA treatments were adapted from a basic procedure previously developed by Nocker and Camper (2006). Briefly, stock solutions of EMA and PMA were added to sample tubes containing 500 μl viable or heat-killed cells to reach a final dye concentration of 50 μM for PMA and 25, 50, or 100 μM for EMA. Samples were incubated in the dark for 10 min to allow dye penetration into cells with damaged membranes. Photo-induced crosslinking of PMA or EMA was achieved by exposing the samples to light for 15 min using a photoactivation system (PhAST Blue, GenIUL, Barcelona, Spain) (Agusti et al. 2010; Elizaquível et al. 2012; Fittipaldi et al. 2010; Fittipaldi et al. 2011; Miotto et al. 2012; Sánchez et al. 2012). The samples were subsequently centrifuged at 14,100 x g for 5 min, and the cell pellets were resuspended in 200 μl of PBS. In addition, 500 μl of control samples, viable and heat-killed cells untreated with PMA or EMA, were concentrated by centrifugation and resuspended in 200 μl of PBS.

To choice the best dye concentration, EMA treatments using different concentrations (25, 50 and 100 μM) were performed.

The experiments to optimize the photoactivation step were conducted with an EMA concentration of 25 μM and an incubation time of 10 min. The samples were exposed to different photoactivation treatments: (Treatment 1) photoactivation for 30 min with three on-off intervals of 10 min; (Treatment 2) double photoactivation treatment for 10 min; or (Treatment 3) continuous photoactivation for 15 min. After photoactivation, the samples were centrifuged at 14,100 x g for 5 min, and the cell pellets were resuspended in 200 μl of PBS.

Different dye incubation conditions were evaluated with EMA at a final concentration of 25 μM for 500 μl of viable or heat-killed cell suspensions. Then, the samples were incubated at different times (10, 30, or 60 min) and at two different temperatures (4 ºC or 22 ºC) in the dark, and were subsequently exposed to light for 15 min. The samples were centrifuged at 14,100 x g for 5 min, and the cell pellets were resuspended in 200 μl of PBS.

DNA purification

Genomic DNA was extracted using the E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions. Before DNA isolation, the samples were exposed to a thermal shock (15 min at 99 ºC and 15 min at 0 ºC, two times) to improve cell lysis.

Quantitative PCR protocol

For *C. albicans* quantification, a qPCR procedure previously described by Maaroufi et al. (2003) was adapted. Sample analysis was performed on a LightCycler-1.5 PCR system (Roche Molecular Diagnostics, Manheim, Germany). The reaction mixture contained 10 μl of Fast Start Taqman Probe Master (Roche Molecular Diagnostic,

Mannheim, Germany), 9 µl of DNA sample, 0.2 µM of the universal fungal amplification primers ITS86 (5′- GTGAATCATCGAATCTTTGAAC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3′) (Turenne et al. 1999), and 0.2 µM of the *C. albicans* specific probe CA-FAM (5′-FAM-ATTGCTTGCGGCGGTAACGTCC-TAMRA-3′) (Shin et al. 1999). The qPCR conditions were optimized (data not shown) and were as follows: 10 min at 95 °C for Taq polymerase activation, and 45 cycles of 15 s at 95 ºC, 1 min at 55 ºC, and 1 min at 72 ºC, for DNA amplification.

Standard DNA curve

A standard DNA curve was established using a 2-day *C. albicans* culture. DNA was obtained using the E.Z.N.A. Tissue DNA kit according to the manufacturer's instructions. The initial amount of yeast cells was determined by culturing and by spectrometry measuring fluorescence at 600 nm, to be 2.0 x 10^6 cfu/ml. Consequently, serial logarithmic dilutions of the initial yeast DNA stock solution in Tris buffer (10 mM Tris, pH 8.5) were performed. Standard DNA curve was performed by duplicate on a LightCycler-1.5 PCR system.

Antifungal treatments

Selection of antifungal products

Before testing antifungal treatments by viable-qPCR, the response of *C. albicans* to different drugs (clotrimazole, fluconazole, and the essential oils, *Thymus vulgaris* and *Matricaria chamomilla*) was monitored by spectrometry to determine the optimal antifungal substances and the best cell-drug contact time.

Briefly, in a 96-well microtiter plate, 50 µl of the working yeast suspension were mixed with 100 µl of Sabouraud glucose-broth (Oxoid, Basingstoke, UK) and 50 µl of

antifungal or essential oil solution. Cell viability was continuously quantified spectrophotometrically at 490 nm for 24 h at 37 ºC, using a microplate reader ELISA (GENios-Tecan, Männedorf, Switzerland). 50 µl of the working yeast suspension diluted in 150 µl of Sabouraud glucose-broth and 200 µl of Sabouraud glucose-broth were used as positive and as negative control, respectively.

A standard curve was also constructed using serial 10-fold dilutions of the initial yeast suspension (OD_{600} of 1.6).

Antifungal treatment assays

500 µl of *C. albicans* cell suspension was added to 1000 µl of Sabouraud glucose-broth and 500 µl of antifungal or essential oil solution. The cells were incubated at 37 ºC, and samples were taken after 16 h. Live cell number was determined using viable-qPCR and plate counts.

Plate counts

To determine the actual correspondence between molecular and culture counts, 0.1 ml of appropriate dilutions of samples from viable-PCR optimizations and antifungal treatment assays were spread on duplicate Sabouraud dextrose-agar plates (Oxoid, Basingstoke, UK) the same day that molecular analyses were carried out. Plates were incubated at 37 ºC for 48-72 h. The colony number was determined using a colony counter (IUL, Barcelona, Spain).

Statistical analysis

Mean values and standard deviations were calculated on the basis of two independent experiments, each performed in duplicate. Microsoft Office Excel 2007 (Microsoft

Corporation, Redmond, WA, USA) was used to determine the equations of standard curves, coefficients of variation, and *p*-values; *p*-values less than 0.05 ($p<0.05$) were considered statistically significant.

Results

Standard DNA curve

Using an optimized protocol, a standard curve (y=-3.5146x + 37.665) was obtained by quantifying 10-fold dilutions of the DNA standard by qPCR and plotting the Cp values against the $\log_{10}$ DNA copies. The slope value was -3.51, close to the theoretical optimum of -3.32 (Higuchi et al. 1993), and the correlation coefficient (R_2) was 0.9999. Furthermore, the detection limit was calculated to be 2.0×10^1 cfu/ml. This standard curve was used to quantify viable cells in subsequent studies.

Optimization of the viable-qPCR procedure

Choice of the intercalating dye

The abilities of EMA and PMA to specifically distinguish dead from viable *C. albicans* cells were compared by adding 50 μM of each dye to suspensions of live and heat-dead cells. As shown in Fig. 1 ((a) and (b)), similar results were obtained for viable cells treated with both intercalating dyes. However, with PMA treatment the number of heat-killed cells detected was significantly higher ($p<0.05$) than with EMA treatment, showing less penetration of PMA into dead cells than with EMA (Fig. 1 (a)). Moreover, the greatest differences in Cp values between dye-treated and untreated dead cells ($\Delta Cp_{\text{dead with dye-dead without dye}}$) and between dead and live cells treated with dye ($\Delta Cp_{\text{dead with dye-live with dye}}$) were observed with EMA (Fig. 1 (b)).

These results indicated that EMA treatment was more effective than PMA treatment in discriminating dead from live *C. albicans* cells in the concentration evaluated (50 μM); therefore, EMA was chosen for subsequent experiments.

Optimization of EMA concentration

Different EMA concentrations (25, 50, and 100 μM) were evaluated to determine the best concentration for selective removal of genomic DNA from dead cells without the loss of DNA quantification from viable cell populations.

The effect of EMA on the difference of Cp values between EMA-treated and untreated live cells ($\Delta Cp_{\text{live with EMA–live without EMA}}$) was shown to increase with EMA concentrations of 25 and 100 μM, showing the greatest difference with an EMA concentration of 100 μM (Fig. 1 (d)). Therefore this concentration was more toxic to the live cells than treatment with EMA concentrations of 25 and 50 μM, which produced similar results and no significant differences in the $\Delta Cp_{\text{dead with EMA-live with EMA}}$ (Fig. 1 (c) and (d)). As a result, a dye concentration of 25 μM was chosen for subsequent experiments.

Optimization of EMA treatment

The effects of different photoactivation treatments and different incubation conditions (time and temperature) were studied. There were no significant differences on the EMA-DNA crosslinking (Fig. 1 (e) and (f)) between a photoactivation step with on-off intervals (treatment 1) and the other two treatments, a double photoactivation treatment (treatment 2) and a continuous photoactivation for 15 min (treatment 3). However, significant differences ($p<0.05$) were obtained between treatment 2 and 3 on live cells (Fig. 1 (e) and (f)). With continuous photoactivation (treatment 3), the number of live

cells detected was higher than with the double photoactivation (treatment 2) (Fig. 1 (e)). Furthermore, when treating live cells with one (treatment 3) or two photoactivation treatments (treatment 2), double treatment resulted in a 2-fold increase in the $\Delta Cp_{\text{live with EMA–live without EMA}}$ value compared with the single continuous treatment (Fig. 1 (f)). These results showed the low suitability of treatment 2 compared with treatment 3. Moreover, in comparison to treatment 3, treatments 1 and 2 resulted in no significant increase in $\Delta Cp_{\text{dead with EMA–live with EMA}}$ (Fig. 1 (f)) and no significant increase in the cfu/ml difference between live and dead cells (Fig.1 (e)). All results indicated that the most suitable light treatment was treatment 3.

A short EMA incubation of 10 min was tested along with prolonged incubations of 30 min and 60 min to analyze the effect of EMA exposure times on the efficiency of viable and dead cell differentiation (Fig. 1 (g) and (h)). For viable *C. albicans* cells, there were no statistically significant differences between the different incubation times (Fig. 1 (g)). Conversely, significant differences were observed among the three incubation times in the case of dead cells (Fig. 1 (g)), with results indicating that 10 min and 60 min were the best options. When analyzing the results through the ΔCp value (Fig. 1 (h)), the highest values in $\Delta Cp_{\text{dead with EMA–live with EMA}}$ and in $\Delta Cp_{\text{dead with dye-dead without dye}}$ were obtained for 10 and 60 min of dark incubation. Moreover, similar results were observed in the value of $\Delta Cp_{\text{live with EMA–live without EMA}}$ for the three incubation times. Based on these results, and considering that 60 min is too long, an incubation time of 10 min was selected.

Since temperature plays a role in microbial cell membrane permeability, the effect of the incubation temperature in the viable-qPCR was investigated (data not shown). No EMA toxicity effects and no significant differences were observed between cells incubated with EMA at 4 and 22 ºC.

In summary, the optimal protocol comprised treatment with 25 μM EMA and 10 min of cell incubation in the dark at 22 ºC followed by continuous photoactivation for 15 min. These conditions were applied in the study of *C. albicans* cell viability after antifungal treatment.

Antifungal susceptibility determined by viable-qPCR

The potential use of viable-qPCR to investigate the killing of *C. albicans* ATCC 2091 by antifungal substances was evaluated. The efficacy of clotrimazole, fluconazole, *M. chamomilla*, and *T. vulgaris* were first tested in a preliminary screening by spectrometry (Fig. 2). *C. albicans* ATCC 2091 was shown to be highly sensitive to the action of clotrimazole and *T. vulgaris* because yeast growth was not observed when these drugs were used (Fig. 2). On the other hand, the *C. albicans* ATCC 2091 treated with fluconazole or *M. chamomilla* showed a minimum decrease in cell growth, about 0.5 log units with respect to the *Candida* control (Fig. 2). Taking into account these results, clotrimazole, fluconazole, and *T. vulgaris* were used to assess *C. albicans* ATCC 2091 viability by viable-qPCR after 16 h of exposure to these compounds. As shown in Fig. 3 ((a) and (b)), treatment with *T. vulgaris* showed a 2.5 log unit reduction in the population of *Candida* cells by viable-qPCR, indicating *T. vulgaris* would compromise the membrane integrity. Non-positive results were obtained by cell culture when heat and *T. vulgaris* treatment were used, demonstrating that both treatments completely killed *C. albicans* ATCC 2091 cells. Similar results were observed with viable-qPCR, but to a lesser extent, because the amplification signals from cells treated with heat and *T. vulgaris* were not reduced completely, values were 10^3 and 10^4 cfu/ml, respectively (Fig. 3 (a)). When cells were treated with clotrimazole, the viability reduction

determined by qPCR and cell culture was 1 log unit in both cases; and no reduction was observed when fluconazole was used (Fig. 3 (a)).

Discussion

To our knowledge, this is the first study to demonstrate that viable-qPCR technique, using PMA or EMA as intercalating dye, can be used for the detection and quantification of live *C. albicans* cells. Selective nucleic acid intercalating dyes, such as EMA and PMA, represent one of the most successful recent approaches to detect viable cells (as defined by an intact cell membrane) by PCR (Fittipaldi et al. 2012) and have been effectively evaluated in different microorganisms (Nocker and Camper 2006; Rudi et al. 2005; van Frankenhuyzen et al. 2011). As both dyes have similar structures, comparable results might be expected; however, some studies have showed differences (Andorrà et al. 2010; Cawthorn et al. 2008; Chang et al. 2010; Nam et al. 2011; Nocker and Camper 2009; Pan and Breidt 2007; Wagner et al. 2008). These studies showed that different parameters, such as the dye concentration and the microorganisms analyzed, play an important role in the specificity of both dyes for live and dead cells. EMA was demonstrated that penetrates the membranes of live cells, and the extent of EMA entry into intact cells is species-dependent (Nocker and Camper 2006). Thus, multiple optimizations for viable-qPCR techniques have been reported focusing on EMA concentration, incubation time, and photoactivation time (Andorrà et al. 2010; Flekna et al. 2007; Minami et al. 2010; Nogva et al. 2003; Pan and Breidt 2007; Wang et al. 2009). On the other hand, PMA has been proposed as a more appropriate alternative due to a comparative study showing that PMA is efficiently excluded from cells with intact cell membranes. Its higher selectivity is probably associated with the higher charge of the molecule (Nocker et al. 2006). Moreover, PMA has the advantages of a more

homogeneous performance and a minor cytotoxic effect with different species (Pan and Breidt 2007; Yañez et al. 2011).

Taking into account these previous works, the effectiveness of PMA and EMA in differentiating between live and dead *C. albicans* cells was analyzed in the present study using viable-qPCR. The results showed that both dyes were suitable for the quantification of viable *C. albicans*. However, due to the fact that not significant differences were observed between both dyes when viable cells were treated, and since the ΔCp between live and dead cells were slightly higher (12 Cp of difference) with EMA than with PMA (10 Cp of difference); therefore, EMA was chosen to be more suitable for further viable-qPCR experiments. Similar findings have been reported by Andorrà et al. (2010) in wine yeast. These authors showed differences between the mean Cp values from dead and viable cells of around 5 and 12 Cp for EMA and 6-11 Cp for PMA, and demonstrated the suitability of both dyes in the detection of viable yeast in alcoholic fermentation of wine.

In this study, viable-qPCR conditions were optimized taking into account different variables: EMA concentration, photoactivation treatment, and incubation conditions in the dark, with the aim that EMA cannot penetrate the intact cell membrane and suppresses the qPCR amplification signal from dead cells. The optimal results were obtained when pre-treating the cells for 10 min in the dark with 25 μM EMA followed by continuous photoactivation for 15 min. The EMA treatment optimization results revealed that increasing the dye concentration, the photoactivation time, and the dye incubation time did not result in a higher signal reduction with heat-killed cells. In addition, EMA apparently cannot penetrate through the viable cell membrane; although, different temperatures generate changes in cell membrane fluidity and permeability (Van de Vossenberg et al. 1995). Based on the results of the present study, viable-

qPCR can be used to determine the viability of *C. albicans* when heat treatment is used, and the usefulness of this technique for yeast species was confirmed, as demonstrated previously for some wine yeasts (Andorrà et al. 2010) and *Zygosaccharomyces bailii* cells (Rawstorne and Phister 2009).

Moreover, the suitability of viable-qPCR technique to determine the viability of *C. albicans* treated with antifungal therapies was assessed. Two classes of membrane-active antifungals are commonly used in the treatment of *Candida* infections: the polyenes, which bind to ergosterol in fungal cell membranes, causing membrane disruption, and the azoles, such as clotrimazole and fluconazole, which inhibit synthesis of ergosterol, increase cellular permeability and generate changes in the membrane structure (Graybill 2000). Unfortunately, the overuse of broad-spectrum antibiotics has resulted in the emergence of many resistant infectious pathogens, such as fluconazole-resistant *C. albicans* strains (Brion et al. 2007; Gamacho-Montero et al. 2010; Martínez et al. 2002). Therefore, the search for a new class of therapeutic agents is critical and pressing (Calugi et al. 2011). Natural products, medicinal plants, and their derivatives have been used as new sources of alternative therapies to treat or relieve diseases (Gurib-Fakin 2006). *T. vulgaris* is an essential oil with various potent biological activities, including antimicrobial, antioxidant, and anti-inflammatory activities (Tsai et al. 2011). Numerous studies have demonstrated the irreversible effects of thymol, the main compound of *T. vulgaris*, on the cell wall, cell membrane, and cellular organelles (Rasooli and Owlia 2005; Tsai 2011; van Vuuren et al. 2009).

Two drugs (clotrimazole and fluconazole) and one essential oil (*T. vulgaris*) were used to evaluate the suitability of viable-qPCR for evaluating the response of *C. albicans* ATCC 2091 to antifungal treatment. The results obtained in this study showed that the antifungal effect of *T. vulgaris* was comparable to the effect of heat inactivation;

whereas, there was lower impact on the cell membrane of *C. albicans* ATCC 2091 with clotrimazole and minimum impact with fluconazole. These differences obtained could reflect the different mechanisms of action of these compounds with respect to membrane damage in *C. albicans*. Nevertheless, even though the Cp difference associated with clotrimazole is not as pronounced as the Cp difference associated with *T. vulgaris* or heat inactivation, our results still demonstrate that the viable-qPCR assay is capable of differentiating clotrimazole-treated yeast from viable yeast. In addition, *T. vulgaris* treatment had the strongest effect on the viability of *C. albicans* ATCC 2091 and this effect was similarly observed by spectrometry, viable-qPCR, and the plate count method.

In conclusion, this viable-qPCR approach could be useful as an analytical tool for a quick diagnosis of *C. albicans* in clinical samples. This viable-qPCR method is an appropriate technique for evaluating the adequacy of pharmacotherapies in treating candidiasis by distinguishing between live and dead *C. albicans* cells. Moreover, this method provides a more accurate and reliable means of evaluating *C. albicans* susceptibility, to predict clinical outcome, especially in patients treated with antifungal therapies.

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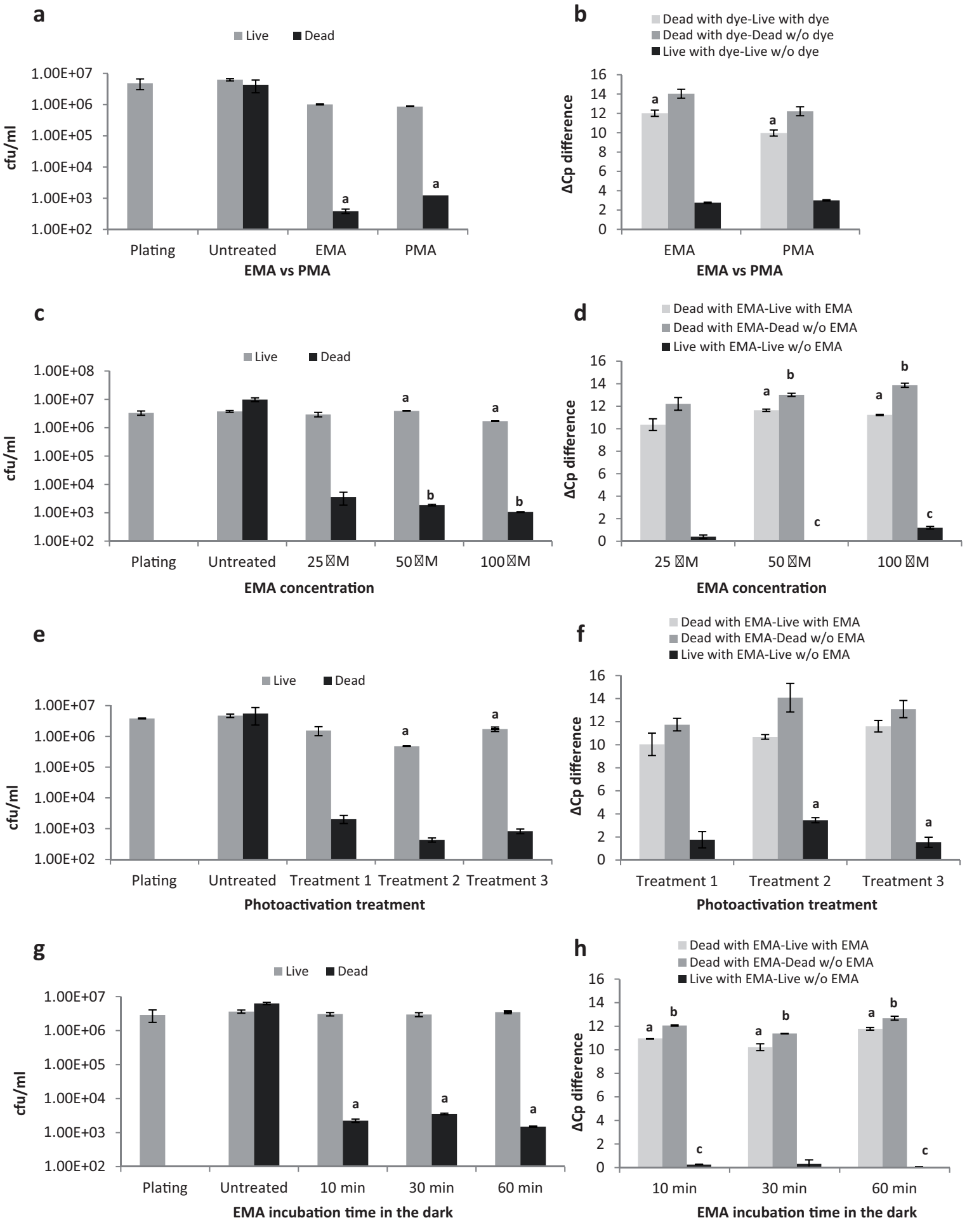
Figure legends

Fig. 1 Effect of EMA or PMA pre-treatment ((a) and (b)), different EMA concentrations ((c) and (d)), different photoactivation treatments ((e) and (f)), and different EMA incubation times ((g) and (h)) on cell number and Cp values obtained from live and heat-killed *C. albicans* cells. Results are presented as absolute cell numbers calculated from qPCR results with the help of a standard curve ((a), (c), (e) and (g)) or as the ΔCp differences ((b), (d), (f) and (h)). Statistically significant differences ($p<0.05$) are indicated by subscript letters. Error bars represent standard deviations obtained from two independent experiments, repeated in duplicate. Treatment 1, photoactivation for 30 min with three on-off intervals of 10 min; Treatment 2, double photoactivation treatment for 10 min; and Treatment 3, continuous photoactivation for 15 min

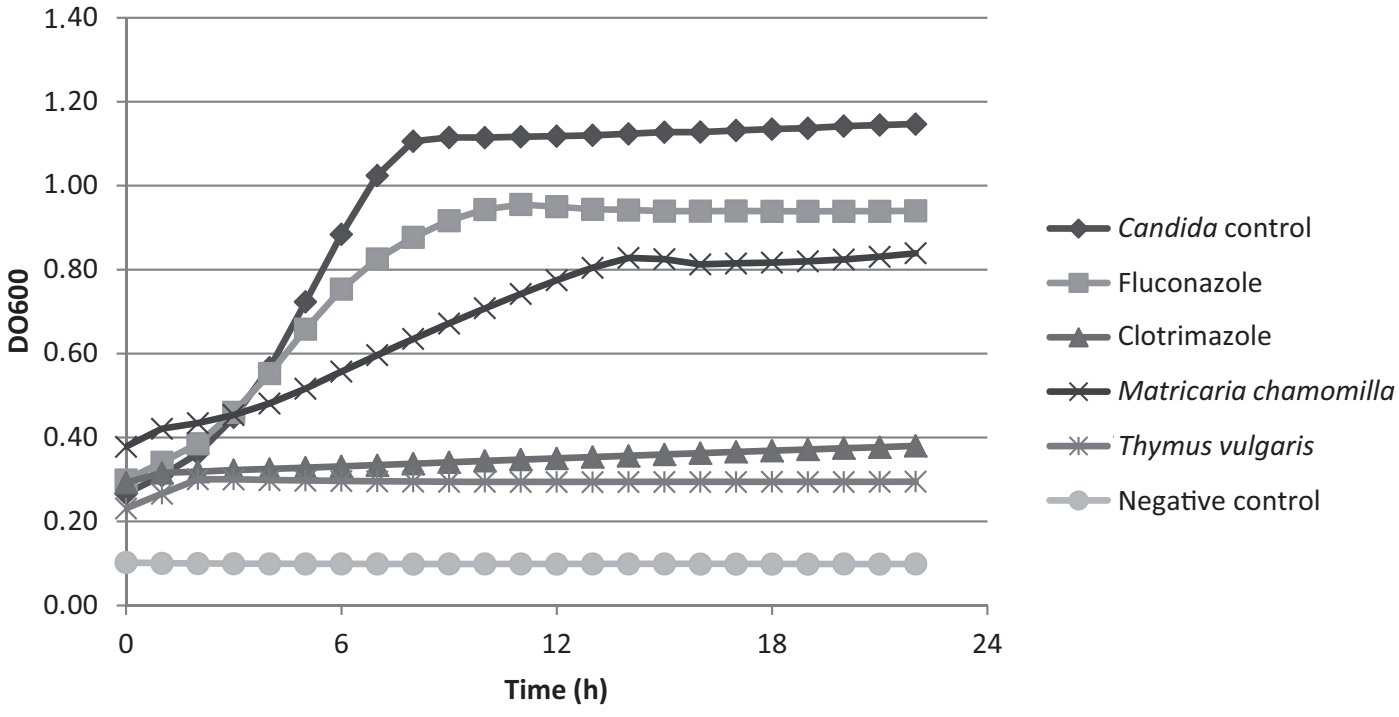
Fig. 2 Growth curve of *C. albicans* treated with clotrimazole, fluconazole, *M. chamomilla,* and *T. vulgaris.* Cell growth was continuously quantified spectrophotometrically at 490 nm for 24 h at 37 ºC. Results shown are the mean values from qPCR assays and are presented as log (cfu/ml) calculated from absorbance results with the help of a standard curve

Fig. 3 Quantitation of the cell viability of *C. albicans* after clotrimazole, fluconazole, and *T. vulgaris* treatment by qPCR and viable-qPCR. The standard deviations from two independent assays repeated in duplicate are shown by bars. Results are presented as cfu/ml, calculated from qPCR results with the help of a standard curve (a) or as the ΔCp differences (b)

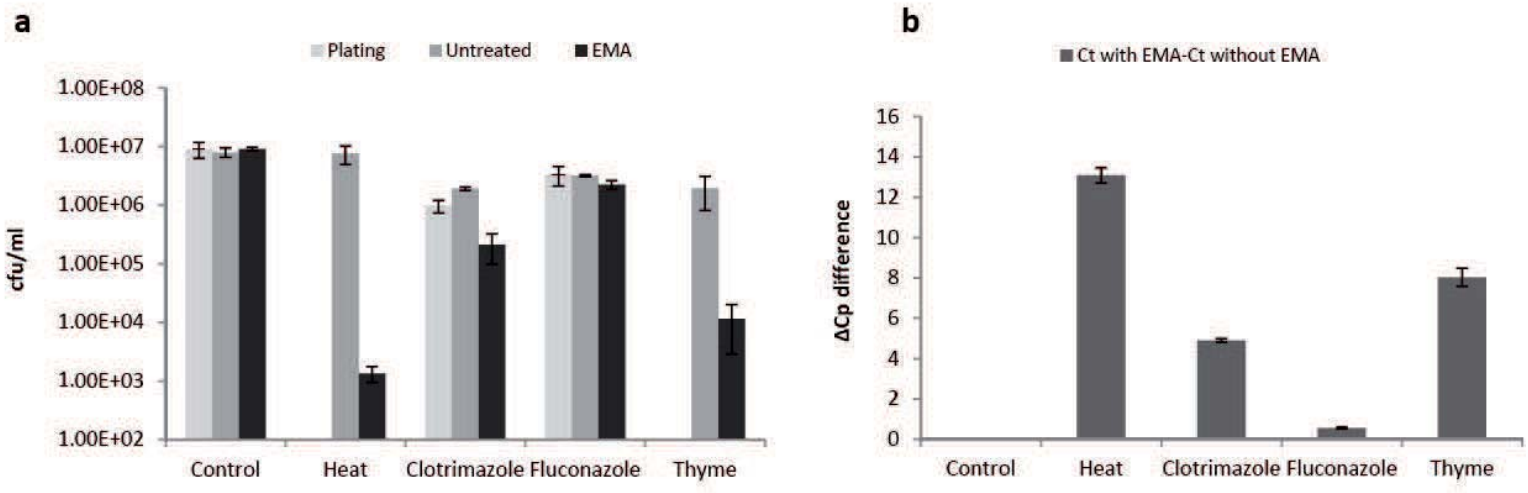
Figure


a
Live
Dead
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
cfu/ml
a
a
Plating
Untreated
EMA
PMA
EMA vs PMA
b
Dead with dye-Live with dye
Dead with dye-Dead w/o dye
Live with dye-Live w/o dye
16
14
12
10
8
6
4
2
0
ΔCp difference
a
a
EMA
PMA
EMA vs PMA
c
Live
Dead
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
cfu/ml
a
a
b
b
Plating
Untreated
25 ⊠M
50 ⊠M
100 ⊠M
EMA concentration
d
Dead with EMA-Live with EMA
Dead with EMA-Dead w/o EMA
Live with EMA-Live w/o EMA
16
14
12
10
8
6
4
2
0
ΔCp difference
a
b
a
b
c
c
25 ⊠M
50 ⊠M
100 ⊠M
EMA concentration
e
Live
Dead
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
cfu/ml
a
a
Plating
Untreated
Treatment 1
Treatment 2
Treatment 3
Photoactivation treatment
f
Dead with EMA-Live with EMA
Dead with EMA-Dead w/o EMA
Live with EMA-Live w/o EMA
16
14
12
10
8
6
4
2
0
ΔCp difference
a
a
Treatment 1
Treatment 2
Treatment 3
Photoactivation treatment
g
Live
Dead
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
cfu/ml
a
a
a
Plating
Untreated
10 min
30 min
60 min
EMA incubation time in the dark
h
Dead with EMA-Live with EMA
Dead with EMA-Dead w/o EMA
Live with EMA-Live w/o EMA
16
14
12
10
8
6
4
2
0
ΔCp difference
a
b
a
b
a
b
c
c
10 min
30 min
60 min
EMA incubation time in the dark

Figure


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DO600
0
6
12
18
24
Time (h)
Candida control
Fluconazole
Clotrimazole
Matricaria chamomilla
Thymus vulgaris
Negative control

Figure


a
Plating
Untreated
EMA
cfu/ml
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
Control
Heat
Clotrimazole
Fluconazole
Thyme
b
Ct with EMA-Ct without EMA
ΔCp difference
16
14
12
10
8
6
4
2
0
Control
Heat
Clotrimazole
Fluconazole
Thyme