Temporal Expression of Genes in Biofilm-Forming Ocular Candida albicans Isolated From Patients With Keratitis and Orbital Cellulitis

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PURPOSE. To study antibiotic susceptibility and biofilm-forming potential of ocular isolates of Candida albicans along with gene expression.

METHODS. Seven clinical isolates of C. albicans (keratitis-6 and orbital cellulitis-1) were evaluated. Biofilm formation in one isolate was monitored by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Expression of 27 genes (real-time PCR) associated with biofilm formation and virulence was compared between biofilm-positive and biofilm-negative ocular C. albicans isolates. The temporal expression (4 to 72 hours) of the 27 overexpressed genes was also determined. Similar studies were also done with biofilm-positive and biofilm-negative nonocular C. albicans.

RESULTS. Four of seven ocular C. albicans isolates exhibited the potential to form biofilm, one of which was resistant to three antifungals, whereas three were susceptible to all. SEM studies indicated that biofilm increased from two to three adherent layers of cells at 24 hours to multiple layers by 72 hours. CLSM showed that biofilm thickness increased from 5.2 μm at 24 hours to 17.98 μm at 72 hours. Upregulation of 27 genes involved in virulence and biofilm formation was observed both in the ocular and nonocular C. albicans positive for biofilm formation and compared to the respective non–biofilm-forming C. albicans. The results also indicated similarity in expression of genes between biofilm-forming ocular and nonocular pathogenic C. albicans. Temporal expression of the 27 genes involved in adhesion, initiation, maturation, and dispersal stages of biofilm in the biofilm-positive ocular isolate indicated that expression pattern followed four different patterns.

CONCLUSIONS. This is the first study showing similarity in expression of genes in biofilm-forming ocular and nonocular isolates of C. albicans, suggesting that upregulated genes could serve as a potential target for developing therapeutic strategies.

Keywords: Candida albicans, biofilm, antimicrobial resistance, expression of genes, ocular yeast, keratitis, orbital cellulitis

Keratitis is an inflammatory disease of the eye wherein the cornea becomes inflamed, making the eye red and painful, and affecting vision. Keratitis is caused by bacteria, fungi, or viruses.1 Several species of fungi like Fusarium solani, Cladosporium spp., Aspergillus fumigatus2–4 are known to be the causative agents of keratitis. C. albicans causes 10% to 45% of fungal ocular infections.1,5,6 Normally, fungi can be treated with antifungal agents (such as voriconazole, fluconazole, caspofungin, and itraconazole), but over time many of these organisms have become resistant to antifungals owing to excessive use of corticosteroids and antibiotics, due to diseases associated with immunodeficiency and the ability of fungi to form biofilms on the surface of contact lenses, intraocular lenses, scleral buckles, and suture material.8,9 Biofilm formation has also been detected in patients with infective crystalline keratopathy15; the microorganisms involved were C. albicans11 and other microorganisms12 such as Streptococci and unidentified gram-negative bacilli and yeast.13 Biofilm-associated strains are known to be more resistant to antimicrobial agents.4,14 Thus, it is the right time to devise strategies to overcome antifungal resistance that may help achieve better treatment outcome.

The potential to form biofilms has been demonstrated in some ocular fungi (A. fumigatus, C. albicans, F. solani, Cladosporium sphaerospermum, and Acremonium implicatum),15–17 but the molecular mechanism of biofilm formation with respect to the genes involved is not established. For instance, it is known that during biofilm formation in nonocular pathogenic C. albicans, genes coding for motility, adhesion to substratum,28–29 efflux pumps,30,31 transcription factors,18–20 virulence,31–33 EPS production, among others, are upregulated. Thus, it would be worthwhile to investigate whether similar genes are upregulated in ocular C. albicans organisms, which exhibit resistance to antimicrobial agents and also exhibit the capability to form biofilm. Our knowledge about ocular isolates of fungi with respect to biofilm formation and antibiotic
Temporal Gene Expression in C. albicans Biofilm

Table 1. Clinical Details of Patients With Candida albicans Infection

<table>
<thead>
<tr>
<th>Laboratory No.</th>
<th>Age/ Sex</th>
<th>Diagnosis</th>
<th>Visual Acuity at Presentation</th>
<th>Medical Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-726/11</td>
<td>60/F</td>
<td>Keratitis</td>
<td>PL + PR accurate</td>
<td>Natamycin</td>
</tr>
<tr>
<td>L-504/12</td>
<td>67/M</td>
<td>Orbital cellulitis</td>
<td>PL + PR inaccurate</td>
<td>KETOZOLE</td>
</tr>
<tr>
<td>L-534/13</td>
<td>66/M</td>
<td>Keratitis</td>
<td>HM + PL + PR accurate</td>
<td>Nil</td>
</tr>
<tr>
<td>L-1376/13</td>
<td>50/M</td>
<td>Keratitis</td>
<td>20/100</td>
<td>Chlorocol</td>
</tr>
<tr>
<td>L-664/14</td>
<td>56/M</td>
<td>Keratitis</td>
<td>CFCF</td>
<td>Nil</td>
</tr>
<tr>
<td>L-2033/14</td>
<td>65/M</td>
<td>Keratitis</td>
<td>CFm2</td>
<td>Nil</td>
</tr>
<tr>
<td>L-391/15</td>
<td>29/F</td>
<td>Keratitis</td>
<td>PL + PR inaccurate</td>
<td>Nil</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Topical</th>
<th>Systemic</th>
<th>Final Visual Acuity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natamycin</td>
<td>KETOZOLE</td>
<td>PL + PR inaccurate</td>
</tr>
<tr>
<td>Nil</td>
<td>Nil</td>
<td>PL + PR inaccurate</td>
</tr>
<tr>
<td>Natamycin</td>
<td>Nil</td>
<td>HM + PR accurate</td>
</tr>
<tr>
<td>Chlorocol</td>
<td>Nil</td>
<td>CFCE PL + PR inaccurate</td>
</tr>
<tr>
<td>Natamycin</td>
<td>Nil</td>
<td>CF/1/2m</td>
</tr>
<tr>
<td>Natamycin</td>
<td>KETOZOLE</td>
<td>HM + PR inaccurate</td>
</tr>
<tr>
<td>Natamycin</td>
<td>Nil</td>
<td>HM + PR accurate</td>
</tr>
</tbody>
</table>

CF: counting fingers; CFCE: counting fingers close to face; F: female; HM: hand movement; M: male; m: meter; PL: perception of light; PR: perception of rays.

Resistance is very limited. With this in view, in the present study we chose ocular C. albicans derived from patients with keratitis and orbital cellulitis to assess the biofilm formation potential and correlation with antifungal sensitivity and to evaluate by real-time PCR whether genes overexpressed during biofilm formation in nonocular pathogenic C. albicans are also overexpressed in ocular C. albicans with a potential to form biofilm.

This would add to our understanding of ocular C. albicans with respect to biofilm formation and identification of targets for overcoming antifungal resistance.

Materials and Methods

Isolates of C. albicans

Seven isolates of C. albicans, obtained from patients diagnosed with microbial keratitis and orbital cellulitis, and attending the outpatient department of the L V Prasad Eye Institute, Hyderabad, India, were used. All the cultures were grown in Sabouraud dextrose medium (SDM) (dextrose 20 g and peptone 10 g in 1 L distilled water and final pH adjusted to 5.6) at 30°C. Clinical features of patients from whom the seven isolates of C. albicans were isolated are shown in Table 1.

Antifungal Susceptibility Profile of Ocular C. albicans

Antibiotic susceptibility of all isolates was performed in compliance with CLSI (Clinical and Laboratory Standards Institute) guidelines, using the broth dilution method44 for natamycin (NA)5 and E-test36 (BioMérieux, Craponne, France) for amphotericin B (AB), voriconazole (VO), itraconazole (IT), caspofungin (CS), and fluconazole (FL), using Mueller Hinton agar plates containing 2% glucose with 0.005% methylene blue (Himedia, M1825, Mumbai, India). Antibiotic profile pattern of ocular C. albicans isolates was classified as susceptible or resistant as based on the guidelines of CLSI (M27-S4). Minimum inhibitory concentration (MIC) breakpoints for itraconazole and natamycin (not available in CLSI guidelines) were noted as per published literature.36

Biofilm-Forming Potential of Ocular Isolates of C. albicans by the Crystal Violet Method

The potential to form biofilm was assessed for all isolates by using the microtiter/tissue culture plate (TCP) method.37,38 Briefly, overnight culture was adjusted to 0.5 McF units (5 × 10^6 cells/mL) with SDM, and 100 μL of this inoculum was added to a single well of a 96-well tissue culture plate (ThermoFisher Scientific, Nunclon, Roskilde, Denmark) already containing 100 μL SDM medium. The plates were incubated at 30°C for 48 hours. After incubation, the broth was discarded and wells were washed thrice with 200 μL phosphate-buffered saline (PBS) (Sigma-Aldrich Corp., St. Louis, MO, USA). Cells that adhered to the plate were stained with 200 μL 0.1% crystal violet (Sigma-Aldrich Corp.) for 30 minutes and washed thrice with 200 μL PBS. The crystal violet that had stained the biofilm was solubilized by adding 200 μL absolute alcohol and the absorbance of the crystal violet solution was spectrophotometrically monitored at 655 nm39 (iMARK microplate reader; Bio-Rad, Shinagawa-ku, Tokyo, Japan). C. albicans ATCC90028, which is biofilm negative, was used as control. The absorbance (OD) value of the control culture (C. albicans ATCC90028) at 655 nm, following crystal violet staining and washing, was 0.25 and all isolates that had an OD value greater than 0.25 were considered as biofilm-positive strains of C. albicans. All experiments were set up in triplicate with incubation at 30°C for 2 days.

The optimum temperature and pH for biofilm formation was also determined for ocular C. albicans by using SDM medium of different pH (3.6, 5.6, and 7.6) and incubation at different temperatures (25°C, 30°C, and 37°C).

Biofilm-Forming Potential of Ocular Isolates of C. albicans by the XTT Method

Biofilm formation in seven ocular isolates of C. albicans and two ATCC isolates (ATCC 90028 and ATCC 14053) was also determined by the XTT ((2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) method.40 Briefly, overnight culture was adjusted to 100 McF units (5 × 10^6 cells/mL) by using SDM (pH 5.6) and then transferred to one of the wells of the 96-well tissue culture plate (ThermoFisher Scientific, Nunclon) already containing 100 μL SDM (pH 5.6). The plate was incubated at 30°C for 48 hours. After incubation, the broth was discarded and wells were washed thrice with 200 μL PBS (Sigma-Aldrich Corp.). Next, 200 μL PBS with 50.5 μL 1 mg/mL XTT (Sigma-Aldrich Corp.) and 2.5 μL 0.4 mM of Menadione (Sigma-Aldrich Corp.) were added to each well of the 96-well plates. The plates were incubated in the dark at 37°C for 3 hours. One hundred microliters of solution from each well was then transferred to the wells of a new plate and calorimetric changes were measured at 490 nm by using a microplate ELISA reader (SpectraMax M3; Molecular Devices, Sunnyvale, CA, USA). OD of C. albicans ATCC90028, a biofilm-negative isolate, was used to...
Expression of Candidate Genes in Ocular C. albicans L-391/2015 (Biofilm Positive), Ocular C. albicans L-1376/2013 (Biofilm Negative), Nonocular C. albicans ATCC14053 (Biofilm Positive), and Nonocular C. albicans ATCC 90028 (Biofilm Negative)

RNA was extracted from C. albicans L-391/2015 in the biofilm phase by allowing the organisms to grow on a Petri plate for 48 hours at 30°C as described above. After 48 hours of growth, planktonic cells were decanted and the biofilm was washed thoroughly with PBS. Cells adherent to the Petri plate were removed by using a cell scraper (ThermoFisher Scientific, Nunclon) and collected directly in RNA later (Ambion, Carlsbad, CA, USA) and incubated for 30 minutes. RNA was then extracted by using Qiagen (Hilden, Germany) RNA isolation kit and converted into cDNA (Takara Prime script 1 strand cDNA synthesis kit; Takara BIO, Inc., Nishihigashi, Shiga, Japan). Quality and quantity of RNA were checked by A260/A280 value of 1.8 to 2.0 and by visualization of the RNA after electrophoresis on 1.5% agarose gel. RNA was also extracted from non-biofilm-forming ocular C. albicans L-1376/2013 (as a control), which was allowed to grow for 48 hours at 30°C. To compare the expression of genes between ocular and nonocular C. albicans, RNA was also extracted from nonocular C. albicans ATCC 14053 (biofilm positive) and nonocular ATCC 90028 (biofilm negative).

RT-PCR was then used for monitoring the expression of 27 different genes coding for adhesion (ALS1, HW1P1, ECE1, EAP1, and OCH1) and transcription factors required for biofilm formation (EF1α, ACE2, CPH1, TUP1, and ZAP1) and virulence genes coding for transferase activity (MNT4 and MNT2), drug transmembrane transport (MDR1), aspartyl proteinases (SAP1, SAP2, SAP3, SAP4, SAP5, SAP6, SAP7, SAP8, and SAP9), phospholipases (PLB1, PLB2, PLC, and PLD), and ATPases (PMRT1). The primers used for the above genes were as reported for nonocular pathogenic C. albicans (Table 2) and amplification conditions were as follows: 50°C for 2 minutes, 40 cycles (95°C for 30 seconds, 60°C for 1 minute). The results were compared between C. albicans L-391/2015 and C. albicans L-1376/2013 to identify the differential regulation of genes during biofilm formation. Relative expression of genes was calculated by the ΔΔCT method. Only genes that showed >2-fold expression with a P value < 0.05 were considered as significantly differentially expressed. Expression of EF1α gene coding for elongation factor B was used as an internal standard. All values reported represent the mean of three independent experiments.

Expression of genes in nonocular C. albicans ATCC 14053 (biofilm positive) and ATCC 90028 (biofilm negative) was also determined as above. Finally, the gene expression was compared between the biofilm-positive ocular and nonocular strains of C. albicans.

Temporal Expression of Candidate Genes in C. albicans L-391/2015 (Biofilm Positive) During Biofilm Formation

RNA was extracted from biofilm phase of C. albicans L-391/2015 incubated for 4, 12, 24, 48, and 72 hours and used as the source of cDNA preparation. Biofilm cells were grown as in the abovementioned protocol, and RNA was isolated and converted into cDNA. The expression of 27 genes was monitored by RT-PCR to evaluate the temporal expression of the genes during biofilm formation. Expression of cells harvested after 4 hours was used as a control, since biofilm formation was not visible at this time.
RESULTS

Antibiotic Susceptibility and Biofilm Formation in Ocular C. albicans

Three of seven isolates of C. albicans, namely, L-391/2015, L-726/2011, and L-2033/2012, were resistant to one or more of the six antifungal drugs tested. Biofilm-forming potential was assessed by using the microtiter/TCP and XTT methods. The results indicated that four of seven isolates of ocular C. albicans (L-391/2015, L-534/2013, L-664/2014, and L-2033/2014) were positive for biofilm formation by both the crystal violet and XTT methods.

### Table 2. Primers Used for RT-PCR of Genes Involved in Biofilm Development in Ocular C. albicans

<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Gene</th>
<th>Sequence (5'-3')</th>
<th>Molecular Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EFG1</td>
<td>F- TGCCAATAATGTTGTCCGTGGTG&lt;br&gt;R- CCCATCTCTTCTACACACCTGCACAAATT</td>
<td>Transcription</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>ACE2</td>
<td>F- AGAATTGACCGGGGCAGATGT&lt;br&gt;R- ARTGGGGAATCTGCCGACAGCAGAG</td>
<td>Transcription</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>ECE1</td>
<td>F- GTCGTTAGATGCTGGAGTAT&lt;br&gt;R- TTTTGTCCGAGGTCAGATG</td>
<td>Adhesion</td>
<td>56,68</td>
</tr>
<tr>
<td>4</td>
<td>EAP1</td>
<td>F- TGCTGATGGCTGGACAGGAC&lt;br&gt;R- GCAGACCTGTTCTACGCAAA</td>
<td>Cell wall-related proteins</td>
<td>21,56</td>
</tr>
<tr>
<td>5</td>
<td>HWP1</td>
<td>F- CGGAATCTCTAGTCGCTCCCTCT&lt;br&gt;R- CGACACTGTTGAATTTGGAAGGCTGGCAGATG</td>
<td>Cell wall-related proteins</td>
<td>21,56</td>
</tr>
<tr>
<td>6</td>
<td>SAP1</td>
<td>F- GAAACCAAGGATTTATGCGCAAAG&lt;br&gt;R- TTGTCCGAGGTCAGATG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>7</td>
<td>SAP2</td>
<td>F- TCGTTTGTTAAAACAGCGAGGTCCAT&lt;br&gt;R- TATTTGTGCGGATCTGGAACAG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>SAP3</td>
<td>F- CAGCTCTGGATTTACGTCCTCATT&lt;br&gt;R- TCCAAAAAAAGAAGATGACATTCGATGCAATCA</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>SAP4</td>
<td>F- CGCTGTTGCTTATAGATGATG&lt;br&gt;R- AGGATGTAATACGTCAGCAGAGGA</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>SAP5</td>
<td>F- CACAGATTTCCCACGCAACT&lt;br&gt;R- TTATTGTGCGGATCTGGAACAG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>11</td>
<td>SAP6</td>
<td>F- GAATTGAAAACTCCATCGGACGATG&lt;br&gt;R- CAGACGAGGAGGAGGAGGAGG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>12</td>
<td>SAP7</td>
<td>F- TTCTCTGGATGGCTGGAGTAT&lt;br&gt;R- AATGTTCGGATCTGGAACAG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>13</td>
<td>SAP8</td>
<td>F- TCGTTTGTTAAAATGCGAGGTCCAT&lt;br&gt;R- TATTTGTGCGGATCTGGAACAG</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>14</td>
<td>SAP9</td>
<td>F- TTGGGATTTGACGACAAACATCT&lt;br&gt;R- GCTGAATGACGTGTGCTGGTA</td>
<td>Aspartyl proteases</td>
<td>21</td>
</tr>
<tr>
<td>15</td>
<td>PLB1</td>
<td>F- GGTGGAGGAGATGTCGACA&lt;br&gt;R- ACGACTTACGTGATATGCGAACA</td>
<td>Phospholipase</td>
<td>21</td>
</tr>
<tr>
<td>16</td>
<td>PLB2</td>
<td>F- TGGACCTTTGGGCGGACAA&lt;br&gt;R- GGCGCGTCGTTTGTGAA</td>
<td>Phospholipase</td>
<td>21</td>
</tr>
<tr>
<td>17</td>
<td>PLC</td>
<td>F- AGCCACCAATTGGCCCAACTTA&lt;br&gt;R- ACCTGTTGATTAAAAGGATGTTTCCC</td>
<td>Phospholipase</td>
<td>21</td>
</tr>
<tr>
<td>18</td>
<td>PLD</td>
<td>F- TGTTACCTGGTGAAGGTTGGA&lt;br&gt;R- CAGACGAGGAGGAGGAGGAGG</td>
<td>Phospholipase</td>
<td>21</td>
</tr>
<tr>
<td>19</td>
<td>CPH1</td>
<td>F- AGCCACCAAGGCTCTTACT&lt;br&gt;R- GCTGTTGCTTACGAGGAGGAGG</td>
<td>Phospholipase</td>
<td>21</td>
</tr>
<tr>
<td>20</td>
<td>ZAP1</td>
<td>F- CGAATTCAAAACCCACAGCTTTCATT&lt;br&gt;R- CCCCTGTTGCTCATGGTTGTTT</td>
<td>Transcriptional factor</td>
<td>70</td>
</tr>
<tr>
<td>21</td>
<td>ALS1</td>
<td>F- CCAAATCTGACTAAGACTGTCAC&lt;br&gt;R- ACAATTCGATTTGGCGTACGAA</td>
<td>Zinc response regulator</td>
<td>55</td>
</tr>
<tr>
<td>22</td>
<td>MDR1</td>
<td>F- TGCTCGCGATTGCCTGAGAA&lt;br&gt;R- GCAGTGGGATTTGGCAGATG</td>
<td>Major facilitator transporter</td>
<td>69,72</td>
</tr>
<tr>
<td>23</td>
<td>MNT2</td>
<td>F- CGTCAAGGAGGAGGAGGAGG&lt;br&gt;R- GAGGAGGAGGAGGAGGAGG</td>
<td>Glycosyltransferase</td>
<td>55</td>
</tr>
<tr>
<td>24</td>
<td>MNT4</td>
<td>F- TGAGCAGGAGGAGGAGGAGG&lt;br&gt;R- GAGGAGGAGGAGGAGGAGG</td>
<td>Glycosyltransferase</td>
<td>55</td>
</tr>
<tr>
<td>25</td>
<td>PMR1</td>
<td>F- GAACCTTTGGGCGGAGGAGG&lt;br&gt;R- GAGGAGGAGGAGGAGGAGG</td>
<td>ABC transporter</td>
<td>55</td>
</tr>
<tr>
<td>26</td>
<td>OCH1</td>
<td>F- TCTCATTGTTGTTGCGTTG&lt;br&gt;R- TCTAGACATGGACACCTGCACT&lt;br&gt;T</td>
<td>Membrane protein</td>
<td>55</td>
</tr>
<tr>
<td>27</td>
<td>TUP1</td>
<td>F- GCTCGAGTAAACCCAGTTGAG&lt;br&gt;R- CTTCGGTCTTCTCTTTGAGGTTT&lt;br&gt;T</td>
<td>Transcriptional repressor</td>
<td>69</td>
</tr>
<tr>
<td>28</td>
<td>EFB1</td>
<td>F- AAAGAGGCTGCTTAAAGAGGAGG&lt;br&gt;R- GGGCCTGAGGTTTGTGAA</td>
<td>Housekeeping gene</td>
<td>21</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
to three and two antifungals, respectively, and L-534/2013 and L-664/2014, which were susceptible to the six antifungals (Table 3).

**Table 3. Susceptibility of Seven Ocular C. albicans Isolates to Six Different Antifungal Agents**

<table>
<thead>
<tr>
<th>Laboratory Number</th>
<th>AB</th>
<th>IT</th>
<th>VO</th>
<th>CS</th>
<th>FL</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-726/2011</td>
<td>0.094 (S)</td>
<td>8 (R)</td>
<td>0.25 (S)</td>
<td>0.38 (S)</td>
<td>128 (R)</td>
<td>8.00 (S)</td>
</tr>
<tr>
<td>L-304/2012</td>
<td>0.094 (S)</td>
<td>0.5 (S)</td>
<td>0.064 (S)</td>
<td>0.064 (S)</td>
<td>2 (S)</td>
<td>8.00 (S)</td>
</tr>
<tr>
<td>L-534/2013</td>
<td>0.125 (S)</td>
<td>0.5 (S)</td>
<td>0.016 (S)</td>
<td>0.064 (S)</td>
<td>2.00 (S)</td>
<td>4.00 (S)</td>
</tr>
<tr>
<td>L-1376/2015</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.032 (S)</td>
<td>0.125 (S)</td>
<td>1.00 (S)</td>
<td>8.00 (S)</td>
</tr>
<tr>
<td>L-664/2014</td>
<td>0.25 (S)</td>
<td>0.19 (S)</td>
<td>0.023 (S)</td>
<td>0.052 (S)</td>
<td>2.00 (S)</td>
<td>8.00 (S)</td>
</tr>
<tr>
<td>L-2033/2014</td>
<td>0.094 (S)</td>
<td>0.75 (R)</td>
<td>0.094 (S)</td>
<td>0.125 (S)</td>
<td>16 (R)</td>
<td>16 (S)</td>
</tr>
<tr>
<td>L-391/2015</td>
<td>0.38 (S)</td>
<td>16 (R)</td>
<td>8 (R)</td>
<td>2 (R)</td>
<td>4.00 (S)</td>
<td>16 (S)</td>
</tr>
</tbody>
</table>

R, resistant; S, susceptible.

* Values above are in μg/mL. MIC of the six antibiotics as per the CLSI guidelines in μg/mL are AB ≤ 1, IT ≤ 0.5, VO ≤ 0.75, CS ≤ 0.5, FL ≤ 4, and NA ≤ 16. This included L-391/2015, which was resistant to three antifungals, and L-534/2013, L-664/2014, and L-2033/2014, which were susceptible to the six antifungals.

**Biofilm Formation in C. albicans L-391/2015**

Monitored by SEM and CLSM

Biofilm formation in C. albicans L-391/2015 grown on an OHP sheet at 30°C for 24, 48, and 72 hours when processed for SEM indicated progressive deposition of biofilm, from two to three adherent layers of cells at 24 hours (Fig. 2A) to a multilayered (8–10 layers) structure by 72 hours (Fig. 2C). CLSM also confirmed luxuriant growth of biofilm by 72 hours of incubation (Fig. 3A) and concurrently, the thickness increased from 4.65 μm at 4 hours to 17.98 μm at 72 hours (Fig. 3B), after which the thickness decreased at 96 hours. It may also be noted that the clumping of cells seen from 24 to 96 hours is also facilitated by the extra polymeric secretion, which masks the morphology of the cells.

**Antifungal Susceptibility of C. albicans L-391/2015 Before and After Biofilm Formation**

Antifungal susceptibility of C. albicans L-391/2015 to six different antifungal drugs was monitored before and after biofilm formation. In general it was observed that the MIC of an antifungal agent required to kill C. albicans L-391/2015 in the planktonic phase was 100 to 200 times less than the MIC for biofilm cells. For instance, the MIC of AB was 1 μg/mL before biofilm formation (Fig. 4A) and in the biofilm phase it increased to 200 μg/mL (Fig. 4B); in the same manner, the MIC was as follows for IT (16 μg/mL and 3.2 mg/mL before and after biofilm formation, respectively), CS (2 μg/mL and 0.2 mg/mL), NA (16 μg/mL and 1.6 mg/mL), and VO (8 μg/mL and 0.8 mg/mL) (Figs. 4A, 4B).

**Expression of Candidate Genes in C. albicans L-391/2015 (Biofilm Positive) and C. albicans L-1376/2013 (Biofilm Negative)**

The purpose of the study was to compare the expression of 27 genes associated with biofilm formation and virulence between biofilm-positive ocular C. albicans and biofilm-positive nonocular C. albicans. For this purpose, by RT-PCR we determined the genes that were up- or downregulated in the ocular biofilm-forming C. albicans L-391/2015 by using ocular C. albicans L-1376/2013 (biofilm negative) as the control; we also determined the expression of the 27 above genes in nonocular biofilm-forming C. albicans (ATCC 14053) by using the nonocular biofilm-negative C. albicans (ATCC 90028) as the control. The results indicated that all the 27 genes were...
significantly overexpressed in the ocular biofilm-forming C. albicans in the biofilm phase (L-391/2015) as compared to their respective controls (L-1376/2013). In the nonocular C. albicans ATCC 14053 in the biofilm phase, 24 genes were upregulated as compared to the respective control (ATCC 90028). The remaining three genes were neither up- nor downregulated in the nonocular biofilm-forming C. albicans as compared to the control (Table 4). The three genes that were not overexpressed in the nonocular C. albicans in the biofilm phase include ACE2, CPH1, and SAP6. Only genes that showed >2.0 fold-increase in expression with a P value < 0.05 were considered as significantly differentially expressed.

Temporal Gene Expression During Different Stages of Biofilm Formation in C. albicans L-391/2015

Time-dependent expression of 27 of the above genes encoding for biofilm formation, drug resistance, transcription factors, adhesion, and virulence was monitored in C. albicans L-391/2015 by real-time PCR using cells grown for 4 hours as a control, since at 4 hours no biofilm formation was detected (Fig. 5). In biofilm cells, the expression of genes varied depending on the gene and the time of biofilm formation (12–72 hours). The expression followed four different patterns. In pattern 1, five genes, namely, SAP3, SAP7, PLD, PLB1, ALS1,
and ACE2, showed no significant change in expression between 12 and 72 hours as compared to the 4-hour cells (Fig. 5A). In pattern 2, four genes, namely, PMR1, SAP1, TUP1, and SAP2, showed more than 2.0-fold expression between 12 and 72 hours (Fig. 5B). In pattern 3, eleven genes, namely, HWPI, PLC, OCH1, MNT4, MDR1, PLB2, SAP5, EFG1, EAP1, MNT2, and ECE1, showed more than 2.0-fold expression at up to 48 hours and increased significantly at 72 hours (\(P \leq 0.05\)) (Fig. 5C). In pattern 4, six genes, namely, ZAP1, SAP8, SAP4, SAP6, and CPH1, showed increased expression from 24 to 72 hours. All the upregulated genes showed more than 2-fold increases in expression with a \(P\) value \(\leq 0.05\) as compared to the expression in 4-hour cells (Fig. 5D). Thus, it is obvious that genes that are required for biofilm formation, drug resistance, and virulence exhibit differential temporal expression patterns in ocular C. albicans L-391/2015.

**DISCUSSION**

Our results indicate that of seven ocular isolates of C. albicans, four were biofilm positive and only two of these biofilm-positive isolates were resistant to at least two or more of the six antifungals tested. The other two isolates, namely, C. albicans L-534/2013 and L-664/2014, were sensitive to all the six antifungals tested. We also observed increased expression of glycoltransferase genes MNT2 and MNT4\(^{34,37}\) and PMR1, the ABC transporter gene.\(^3\) We also compared increased expression of the above 27 genes (Table 4) in ocular C. albicans and nonocular C. albicans in the biofilm phase and observed that 24 of the 27 genes were overexpressed in both strains of C. albicans in the biofilm phase. Only three genes (ACE2, CPH1, and SAP6) were neither up- nor downregulated in the nonocular biofilm-forming C. albicans. Other genes with similar function may be compensating for their activity. Overall, these studies would indicate that expression of genes related to biofilm formation and antimicrobial resistance are similar in ocular and nonocular C. albicans.

Earlier studies using nonocular isolates of C. albicans have reported that biofilm confers high level of resistance to antifungals, notably the azoles and polyenes. This has been attributed to the elevated expression of several genes\(^{1,15,16}\) like the azole efflux genes CDR1, CDR2, and MDR1\(^{10,20,27}\); the genes that are implicated in adhesion like ALS1, HWPI, EAP1, ECE1, and OCH1\(^{18–25}\); genes coding for several secreted enzymes crucial for host tissue invasion and nutrient acquisition\(^{11}\) like the secreted aspartyl proteinases (SAPs) and phospholipases (PLB, PLC, and PLD)\(^{31,32,33}\); and several transcriptional regulators like EFG1, TEC1, BCR1, NDT80, BRG1, ROBI, ACE2, ZAP1, and TUP1 required for biofilm development.\(^{38–40}\) Fox et al.\(^{53}\) have demonstrated that three new regulators, FLO8, GAL4, and RFX2, also play distinct roles during biofilm development over time. The present study confirms increased expression of several of the above genes like MDR1, ALS1, HWPI, EAP1, ECE1, and OCH1, as observed in earlier studies\(^{3,5}\) on ocular Candida and non-Candida isolates, showing that non-biofilm-forming isolates are resistant to antifungals. Similar studies on Acinetobacter baumannii have also demonstrated that of 63 strong biofilm makers, 79.4% are non-multidrug-resistant strains.\(^{44}\)

C. albicans L-391/2015 was the best biofilm producer, and the thickness of the biofilm increased with time of incubation from 24 to 72 hours as judged both by SEM and CLSM. However, by 96 hours of growth, biofilm thickness was reduced and appeared to be less dense and more dispersed; in the confocal micrographs, the biofilm at 96 hours revealed single cells of C. albicans, implying the occurrence of biofilm dispersal cells.\(^{25,28,34,35}\) This study also confirms that biofilm-forming cells in the biofilm phase are 100 to 200 times more resistant to antifungals as compared to the planktonic cells. This is in accordance with earlier studies on pathogenic C. albicans\(^{18}\) and other fungi.\(^1,14\)

**FIGURE 4.** Antifungal susceptibility of C. albicans L-391/2015 before (A) and after (B) biofilm formation. In (B) x-axis represents the MIC concentration of the above six antifungal agents at 10, 100, and 200 times the MIC concentration of itraconazole (10X, 100X, and 200X, respectively) as determined in (A). MIC of amphotericin B was 1 \(\mu\)g/mL before biofilm formation (A) and in the biofilm phase it increased to 200 \(\mu\)g/mL (B). (A, B) In the same manner, the MIC was as follows for itraconazole (16 \(\mu\)g/mL and 3.2 mg/mL before and after biofilm formation, respectively), caspofungin (2 \(\mu\)g/mL and 0.2 mg/mL), fluconazole (8 \(\mu\)g/mL and 3.2 mg/mL), natamycin (16 \(\mu\)g/mL and 1.6 mg/mL), and voriconazole (8 \(\mu\)g/mL and 0.8 mg/mL).
then the maturation step up to 72 hours when the yeast cells or hyphae get embedded in an extracellular polymeric matrix and are not clearly visible, and finally, the dispersal phase up to 96 hours when the biofilm appears to be fragmented and a few single cells are visible (Fig. 3A).

Temporal expression of genes during biofilm formation has helped to delineate the expression of genes during the four different phases. According to Finkel and Mitchell, in pathogenic C. albicans, EAP1 and ALS1 are expressed in the adhesion phase to bind to the substrate, ALS3 and HWPI in the initiation phase to mediate cell-cell binding, ZAP1 in the maturation phase characterized by accumulation of extracellular polymeric substance, and the transcription regulators UME6, PES1, and NRG1 are expressed in the dispersal phase when the biofilm releases cells that seed new biofilms. In ocular C. albicans three of the four adhesion genes, namely, HWPI, ECE1, and EAP1, but not ALS, showed significant upregulation at 12 hours and beyond, up to 72 hours, implying these regulate binding to the substratum all through the biofilm phase. Chandra et al., Samaranayake et al., and Zhao et al. have earlier demonstrated that ALS1 was not overexpressed at the adhesion phase. In addition to ALS1, it was observed that five other genes—coding for the secreted enzymes aspartyl proteinases (SAP3 and SAP7), phospholipases (PLD and PLB1), and one involved in transcription (ACE2)—showed less than 2-fold increase in expression up to 72 hours. Though ALS, has been implicated in the adhesion phase, the other four genes—SAP3, SAP7, PLD, PLB1—and ACE2 with similar expression pattern are not involved in adhesion. SAP genes (SAP1–SAP10) are known to be differentially regulated in yeast during biofilm formation, and the SAP genes along with PLB genes have a key role in yeast colonization and contribute to the pathogenesis of C. albicans. In accordance with these early reports in ocular C. albicans, the expression of SAP5, PLC, and PLB2 was also upregulated in the biofilm cells and the genes were significantly overexpressed at 72 hours compared to 12 to 48 hours of biofilm phase, implying that these genes are required in the maturation phase. In addition, the pattern of expression of HWPI, OCHI, MNT4, MDR1, EFG1, EAP1, MNT2, and ECE1 was also similar, implying that these genes are also required in the maturation phase. EFG1, which is a transcription factor and regulates HWPI, also exhibits a similar expression pattern. The increased expression of HWPI in biofilm cells is in accordance with that reported by Samaranayake et al. It is suggested that HWPI is required for filamentation, normal biofilm formation, and virulence. Earlier studies have indicated increased expression of MDR1 and other drug-resistant genes like CDR1, CDR2, and FLU1 in biofilm-associated C. albicans cells compared to planktonic cells.
and in vivo.\textsuperscript{64,65} The expression of four genes, namely, \textit{SAP1}, \textit{SAP2}, \textit{PMR1}, and \textit{TUP1} was upregulated from 12 to 72 hours, implying that they are required from adhesion to maturation phase. An earlier study has demonstrated that \textit{TUP1} is upregulated in farnesol-treated biofilms.\textsuperscript{30} In ocular \textit{C. albicans}, the \textit{ZAP1} gene, which is a Zinc-response transcription factor, was the only gene that showed increased expression all through the biofilm formation phase, with greater increase in expression between 48 and 72 hours of biofilm formation, and may thus be required for maturation phase/dispersal phase. In \textit{C. albicans}, \textit{ZAP1} is a negative regulator of a major matrix component\textsuperscript{54} and is required for efficient hyphae formation.\textsuperscript{56,67}

Our results confirm earlier studies that \textit{ALS1}, \textit{HWP1}, \textit{OCH1}, \textit{MNT4}, \textit{MDR1}, \textit{EFG1}, \textit{ZAP1}, \textit{PLB1}, and \textit{SAP7} are associated with biofilm formation. In addition, this study on ocular \textit{C. albicans} also identified other genes such as \textit{SAP1} and \textit{PMR1} in the adhesion phase; \textit{PLC}, \textit{PLB2}, and \textit{SAP5} for initiation and dispersal phases; and \textit{ACT1}, \textit{PLD}, and \textit{SAP3} all through the biofilm formation phase.

In conclusion, ocular \textit{C. albicans} organisms isolated from patients diagnosed with microbial keratitis and cellulitis exhibit differential susceptibility to antifungals, and some have the potential to form a biofilm. In the biofilm phase \textit{C. albicans} was 100 times more resistant to antifungals than the planktonic phase cells. Several genes associated with biofilm formation, drug resistance, and virulence were upregulated in biofilm-forming \textit{C. albicans} compared to the non–biofilm-forming \textit{C. albicans}. Temporal expression of genes in biofilm-forming \textit{C. albicans} helped to identify potential genes involved in different phases of biofilm formation; such genes could serve as potential targets for overcoming biofilm formation and concurrent resistance to antifungal agents.

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References


Temporal Gene Expression in C. albicans Biofilm


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