Journal of Medical Sciences

ISSN 1682-4474

science alert

ANSInet
an open access publisher
http://ansinet.com

Research Paper

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued six times per year on paper and in electronic format.

For further information about this article or if you need reprints, please contact:

Dr. P.P. Chong
Department of Biomedical Sciences,
Faculty of Medicine
and Health Sciences,
University Putra Malaysia,
43400 Serdang, Selangor,
Malaysia

Fax: (60)-3-89426957
(60)-3-89436178

J. Med. Sci., 6 (5): 713-723
September-October, 2006

Transcriptional and Sequencing Analysis of CtCDR1, CtMDR1 and CtERG11 Genes in Fluconazole-Resistant *Candida tropicalis* Isolates from Candidemia Patients

[1]V.C. Yong, [1]L.M. Chew, [1]C.L. Kwan, [2]K.P. Ng,
[3]H.F. Seow and [1]P.P. Chong

Fluconazole resistance in *Candida albicans* can be caused by several resistance mechanisms which include active drug efflux pumps encoded by CDR1 and MDR1 genes as well as mutations in the drug target enzyme encoded by ERG11. To investigate whether the germ tube-negative species of *Candida tropicalis* have the same mechanisms for fluconazole resistance as the germ tube-positive *Candida albicans*, drug susceptible and resistant *Candida tropicalis* isolates were grown in different fluconazole concentrations and analyzed by reverse transcription polymerase chain reaction (RT PCR). It was shown that the expression of CtCDR1 and CtMDR1 in drug resistant *Candida tropicalis* were upregulated at 2.5 fold for both genes compared to drug susceptible *Candida tropicalis* in fluconazole-containing medium. However, no difference of expression level was observed in ERG11 transcript throughout the induction period with fluconazole. These results demonstrate that efflux pumps play an important role in *Candida tropicalis* resistance towards azole drugs. Interestingly, sequence analysis revealed that several nucleotide substitution mutations were present among the resistant isolates compared to a susceptible ATCC type strain, namely T84P in CtCDR1 gene and F119Y in CtMDR1 gene. However, no point mutations were observed in the ERG11 gene for all the resistant isolates analyzed.

Key words: Azole drug resistance, *Candida tropicalis*, CDR1, ERG11, MDR1

[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 59100 Kuala Lumpur, Malaysia
[3]Department of Clinical Laboratory Sciences,
Faculty of Medicine and Health Sciences,
University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ANSInet
Asian Network for Scientific Information

INTRODUCTION

Candidiasis has gained much attention in recent decades due to its increasing prevalence among immunosuppressive and immunodeficiency patients. In Malaysia, 1114 yeasts isolated from various clinical specimens were examined and 9 species of *Candida* were found namely *C. albicans* (44.2%), *C. parapsilosis* (26.0%), *C. tropicalis* (17.7%), *C. glabrata* (9.6%), *C. krusei* (1.2%), *C. rugosa* (0.6%), *C. guilliermondii* (0.2%), *C. lusitaniae* (0.08%) and *C. kefyr* (0.08%) (Ng *et al*., 1999). Virulent factors which contribute to candidiasis that are under investigations are secreted protease (Zaugg *et al*., 2000), phospholipase (Basu *et al*., 2003), the ability to change growth form (Feng *et al*., 1999) and capsule formation (Sonneborn *et al*., 1999). Thus, the important determinants for fungal infection would be ability to adhere to cell surfaces and potential to penetrate into the cells.

Despite the increasing importance of opportunistic fungal pathogens, the number of effective antifungal drugs remains limited. Usage of amphotericin B is associated with toxicities while therapy with flucytosine is limited due to its bone marrow toxicity and high rate of spontaneous mutation to resistance (Bennett *et al*., 1990). Therefore, to overcome major drawbacks of these antifungal drugs, azole drugs were introduced and it changed the approach of *Candida* sp. infection treatments. Azole drugs are directed against C14α-demethylase in the ergosterol pathway causing ergosterol depletion and accumulation of 14α-demethylsterols (Lupetti *et al*., 2002).

Fluconazole, a water soluble triazole was introduced due to some of its advantageous features including high bioavailability (>90%) after oral administration. Fluconazole is used extensively to treat a wide range of *Candida* infections. Nevertheless, as with ketoconazole, development of resistance to fluconazole was reported due to continuous or intermittent usage over long periods of time in AIDS patients (Odds, 1993).

Fluconazole resistance can be contributed by alterations in sterol biosynthesis, by mutations in the drug target enzyme, sterol 14α-demethylase which reduces affinity to fluconazole (Sanglard and Odds, 2002), or by overexpression of genes coding for membrane transport proteins of the ABC transporters (CDR1) and major facilitator (MDR1) superfamilies. Increased levels of active efflux pumps were accounted to be the prime mechanism of resistance to fluconazole (Lupetti *et al*., 2002). Furthermore, CDR1 and MDR1 transcripts were found upregulated in ketonazole and clotritmazole resistant isolates of *Candida albicans* (Looi *et al*., 2005).

Point mutations in ERG11, such as Y132H, T315A or R476K have been shown to decrease the affinity of the target for azoles (Marichal *et al*., 1999). Threonine-for-isoleucine substitution (I471T) which is not found in the previous study proved to confer azole resistance when overxpressed alone (Kakeya *et al*., 2000). The correlation between resistance and increased mRNA levels of efflux pumps and genetic alterations of ERG11 has been well-documented in several *C. albicans* series (Lyons and White, 2000). Exposure of fungi to the azole drugs resulted in increased level of transcription of ERG11 gene.

Apart from this, a shift of *C. albicans* bloodstream infections to non-*C. albicans* has been observed and can be associated with antifungal prophylaxis or empirical antifungal therapy (Ng *et al*., 2001). Ng *et al*. (1999) also found a high prevalence rate of *Candida tropicalis* at 28.6% in respiratory specimens especially from cancer and immunocompromised patients which eventually cause systemic candidiasis. It is probable that *C. tropicalis* may have greater virulence than other *Candida* species or more opportunity to gain access to blood. *Candida tropicalis* resistance towards fluconazole was correlated with CtCDR1 and CtMDR1 gene expression (Barchiesi *et al*., 2000). In addition to this, it was also associated with rapid *in vitro* acquisition of fluconazole resistance and strongly related to the drug concentration in the medium (Barchiesi *et al*., 2000). However, little is known about the other mechanisms of *C. tropicalis* azole resistance which might be multifactorial and the dynamic development of fluconazole resistance in this *Candida* species.

Thus, a new generation of more potent drugs have to be developed to counter the increasing threat of the emerging azole-resistant *Candida* sp. Therefore, having a better understanding of the mechanism of the *Candida* sp. resistance towards azole drugs is essential so that we can manipulate our knowledge to develop an improved treatment for candidiasis.

MATERIALS AND METHODS

***Candida tropicalis* strains:** The clinical samples of *Candida tropicalis* used in this study originated from the Microbiology Diagnostic Laboratory, University Malaya Medical Centre (UMMC). They were from blood cultures taken from patients with neutropenia. Biochemical tests (API 20C Aux) and microbiological testing were done to confirm that the samples are of *Candida tropicalis* species. Eleven clinical isolates of *Candida tropicalis* which were resistant to fluconazole as well as the *Candida tropicalis* ATCC 750 strain which is fluconazole susceptible were included for this study. These isolates were routinely cultured on Sabouraud's Dextrose Agar

(SDA, Difco), at 37°C for 2-4 days. For liquid culture, isolates were grown in Sabouraud's Dextrose Broth (SDB) at 37°C in a rotary shaker incubator at 200 rpm for 16-48 h.

Species identification: Assimilation of carbohydrate test was carried out for all yeast isolates. For non-albicans isolates, 10 carbohydrates (Glucose, Sucrose, Trehalose, Cellobiose, Arabinose, Galactose, Mannitol, Raffinose, Lactose and Maltose) were used for the test while for *C. albicans* isolates, α-methyl-D-glucoside and xylose were added to the 10 carbohydrates for identifying *C. dubliniensis*.

API 20 C AUX (bioMerieux, sa, France) was used to identify yeast which could not be identified using normal laboratory test format. The yeast was harvested from SDA and resuspended in sterile normal saline and adjusted to turbidity equal to 2 McFarland units. The laboratory procedure by the manufacturer was followed and the yeast identified by using API 20C AUX Analytical Profile Index.

Antifungal drug susceptibility testing: The method was similar to the US-NCCLS M2-A6 disk test method for bacteria (2002), but the Mueller-Hinton agar was supplemented with 2% glucose and 0.5 μg mL^{-1} methylene blue. Fluconazole disks (25 μg, Pfizer) were used in the antifungal susceptibility test. Five isolated colonies on SDA were resuspended in 5 mL normal saline and mixed vigorously. The suspension was adjusted to a turbidity of 0.5 McFarland Standard, which would result in 1-5×10^6 yeast cells per mL. A Mueller-Hinton agar plate was pre-dried at 35°C and inoculated evenly with the inoculum suspension using a cotton swab by cross-streaking. The agar plate was allowed to dry at room temperature for 10 min before a fluconazole disk was applied to the designated position of the plate using a paper template placed under the petri dish. The plates were incubated bottom-up at 35°C for 18 to 24 h. The test results were electronically read by image analysis, interpreted and recorded with a BIOMIC Plate Reader System (Ng *et al*., 2001). Interpretive breakpoints used for fluconazole disk tests were based on zones that correlated with US-NCCLS recommended category breakpoints for the reference broth dilution method; with the interpretive criteria of ≥19 mm = S, 15-18 mm = SDD and ≤14 mm = R.

Induction of gene expression using fluconazole: Fluconazole stock (Pfizer) (2560 μg mL^{-1}) was prepared in 7% DMSO for increased solubility. The solutions were filtered with 0.22 μm Milipore filter and dispensed into several sterile 1.5 mL Eppendorf tubes and stored at -70°C for further usage. In addition, RPMI-1640 medium (Sigma) with 2% glucose buffered with sodium bicarbonate was prepared and filter sterilized.

Then, fluconazole stock solution was serially diluted to obtain the desired fluconazole concentrations namely 128, 64, 32, 16, 8, 4, 2 and 1 μg mL^{-1} in RPMI-1640 medium. The dilution in RPMI medium was done in a sterilized laminar hood and aseptic microbiological techniques were carried out to prevent contamination.

For the induction of resistance gene expression, *Candida tropicalis* ATCC 750 strain which was susceptible to fluconazole and one clinical isolate (MIC 16 μg mL^{-1}) which demonstrated *in vitro* resistance to fluconazole was chosen for this study. After preparation of a 10.0 mL culture medium with fluconazole in 50 mL Falcon tubes, 5 colonies on an SDA plate which had been subcultured at least twice to ensure viability were picked at random using sterile wire loop and were transferred into the 10.0 mL RPMI-1640 (Sigma) + 2% glucose medium containing fluconazole at various concentrations. Then, the tubes were incubated overnight at 37°C in a rotary shaker incubator at 180 rpm.

DNA extraction: After overnight incubation, the yeast cells were harvested by centrifugation at 5000 x g for 10 min, then washed twice with 1.0 mL of PBS. Next, the pellet in the falcon tube was resuspended in 500 μL lysis buffer (50 mM Tris HCl pH 7.5, 10 mM EDTA and 0.5% β-mercaptoethanol). The cell suspension was transferred to a sterile 1.5 mL Eppendorf tube and incubated in a 37°C rotary shaker incubator at 180 rpm for 40 min with constant inversion of the tubes.

SDS and Proteinase K were added into the suspension to final concentration of 1% v/v and 0.4 mg mL^{-1}, mixed gently and the mixture was incubated at 56°C for 60 min. Then, the tubes were boiled at 100°C for 5 min to inactivate the Proteinase K. For removal of residual proteins, RNAs and cell debris, the mixture was extracted with 250 μL of phenol/chloroform/isoamylalcohol (25:24:1). Then, ethanol precipitation was carried out and the DNA pellet was air dried. Finally, the DNA pellet was suspended with 30 μL sterile ultrapure water.

PCR amplification of CDR1 and MDR1: *Candida tropicalis* CDR1 gene was amplified using CtCDR1 forward (5'-CTCTTTTCCTCTCACCTCCAGA-3') and CtCDR1 reverse primer (5'-TGAGACCAGGGGAAGTAA CTGT-3') while *Candida tropicalis* MDR1 gene was amplified using CtMDR1 forward (5'-TCATGTTGGATT CACCCTTC-3') and CtMDR1 reverse primer (5'-TAG

TTCTAGTTGCTGACCATCCAA-3') as shown in Table 1. PCR amplification was performed in 25 μL reaction volumes containing 10 pmol μL^{-1} each forward and reverse primer, 10 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$ and 1 U of Taq Polymerase. For CDR1, initial denaturation step was carried out for 10 min, denaturation step at 94°C for 45 sec, annealing at 57.9°C for 45 sec, elongation at 72°C for 45 sec and repeated for 24 cycles followed by final elongation at 72°C for 10 min. For MDR1, initial denaturation step was carried out for 10 min, denaturation step at 94°C for 45 sec, annealing at 54.9°C for 45 sec, elongation at 72°C for 45 sec and repeated for 24 cycles followed by final elongation at 72°C for 10 min. For ERG11 gene amplification, denaturation step was carried out at 94°C for 45 sec, annealing at 48.9°C for 45 sec, elongation at 72°C for 60 sec and repeated for 29 cycles followed by final elongation at 72°C for 8 min.

On the other hand, ERG11 amplification was done using CgERGf1 (5'-ATGACGGTTTATTTRGGTCC-3') and CgERGr1 (5'-GTGAAGATAGTCATTTCWGGTTG-3') primers. For ERG11 gene, initial denaturation step was carried out for 12 min, denaturation at 94°C for 45 sec, annealing at 48.9°C for 45 sec, elongation at 72°C for 1 min and repeated for 29 cycles followed by final elongation at 72°C for 8 min.

Purification of PCR product: Purification of PCR product from agarose gel was performed using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's protocol. For quantitation of the purified DNA fragment, 1 μL of the purified product fragment and 6 μL of known concentration of DNA molecular weight marker (100 bp ladder, Fermentas) were subjected to gel electrophoresis on a 1.0% (w/v) agarose gel in 1X TAE for 40 min under 75 V. Lastly, the gel was visualized using Alpha Imager, AlphaInnotech and the band intensity was compared against the intensities of the fragments in the DNA molecular weight marker of known concentration.

DNA sequencing: Purified PCR products were subjected to automated DNA sequencing using the ABI PRISM BigDye™ system. The nucleotide sequences obtained were analyzed using BlastN for similarities in nucleotide sequences, BlastX for similarities in protein sequences, ClustalW for multiple sequence alignment and Transeq for nucleotide translations into amino acids.

RNA extraction: In this study, RNeasy Mini Kit (Qiagen) was used to isolate the total RNA from the samples. Prior to harvesting the cells, the culture optical density was checked and adjusted to 0.8 at 600 nm to obtain the mid log phase. Then, the cells were harvested by centrifuging at 1300 rpm for 10 min at 4°C and the supernatant was carefully discarded. Next, 2.0 mL of sterile DEPC-treated water was added and the cells were suspended in the solution before centrifuging again at 1300 rpm for 10 min. The supernatant was carefully removed by pippeting.

Subsequently, the cells were suspended in freshly prepared lysis buffer (50 mM Tris HCl, pH 7.5, 10 mM EDTA, 0.1% β-mercaptoethanol and lyticase) and incubated at 10-30 min at 30°C with gentle shaking in rotary incubator at 100 rpm to generate spheroplasts. Immediately after that, the spheroplasts were centrifuged to pellet it at 500 rpm for 5 min. The supernatant was carefully removed without disturbing the spheroplasts as incomplete removal of the supernatant will inhibit lysis and cause dilution of the lysate. The subsequent protocol was carried out with the Qiagen RNA extraction kit according to manufacturer's recommendations.

Consequently, 5 μL of the extracted RNA mixed with 1 μL of loading dye was loaded into each well of 1% agarose gel made from DEPC treated TAE buffer and run for 40 min at 75 V. The bands were visualized using Alpha Imager.

Reverse transcription and cDNA synthesis: Five microliter of total RNA was added with 1 μL of random hexamer (20 pmol μL^{-1}) and 5 μL of sterile DEPC treated ultrapure water to a total of 11 μL in a 0.5 mL microfuge tube. A negative control which had the same components except MMLV-RT was made to confirm that amplification from PCR originates from conversion of RNA to cDNA and not from DNA itself. The mixture was initially incubated at 70°C for 5 min and then chilled in ice bath for 3 min. The tubes were briefly pulse spun. The master mix consisted of 5X reaction buffer (Promega), 10 mM dNTP (Promega), RNase inhibitor (40 U μL^{-1}) (Promega) and sterile DEPC treated ultrapure water. Eight microliter of the master mix was dispensed into each of the tubes and 1 μL of MMLV Reverse Transcriptase enzyme (Promega) was added into the designated tubes. For negative control, 1 μL of sterile DEPC treated water was added to replace the MMLV reverse transcriptase. The mixture was mixed gently and incubated at 37°C for 1 h. After that, the reaction was terminated by incubation at 70°C for 10 min.

Semi quantitative RT-PCR for cDNA: PCR was performed on the cDNA using gene specific primers. *Candida tropicalis* CDR1 gene was amplified using CtCDR1 forward (5'-CTCTTTTCCTCTCACCTCCAGA-3') and CtCDR1 reverse primer (5'-TGAGACCAGGGGAAGTAA

CTGT-3') while *Candida tropicalis* MDR1 gene was amplified using CtMDR1 forward (5'-TCATGTTGGATT CACCCTTC-3') and CtMDR1 reverse primer (5'-TAGTT CTAGTTGCTGACCATCCAA-3'). ERG 11 amplification was done using CgERGf1 (5'-ATGACGGTTTATTTR GGTCC-3') and CgERGr1 (5'-GTGAAGATAGTCATTT CWGGTTG-3').

In addition to these genes, Actin gene was also amplified as an internal control (housekeeping gene) which has constant expression in all of the isolates. It was amplified using CaActin forward primer (5'-ACCGAAGC TCCAATGAATCCAAAATCC-3') and CaActin reverse primer(5'-GTTTGGTCAATACCAGCAGCTTCCAAA-3'). Actin as housekeeping gene was used to normalize the inconsistency of RNA concentrations among the isolates. For Actin gene, denaturation step was carried out at 94°C for 45 sec, annealing at 52.0°C for 45 sec, elongation at 72°C for 45 sec and repeated for 29 cycles followed by final elongation at 72°C for 10 min.

RESULTS

Antifungal susceptibility test: The MIC values for the clinical isolates of *Candida tropicalis* used in this study are shown in Table 2. Five of the isolates were categorized as resistant to fluconazole according to the disk diffusion method, whereas one isolate was susceptible dose-dependent.

Clustal W CDR1 multiple sequence alignment

```
CDR1-1          TTGAGACCAGGGGAAGTAACTGTTGTTTTAGGTAGACCTGGTGCCGGTTGTTCAACTCTA 60
CDR1-2          TTGAGACCAGGGGAAGTAACTGTTGTTTTAGGTAGACCTGGTGCCGGTTGTTCAACTCTA 60
CDR1-3          TTGAGACCAGGGGAAGTAACTGTTGTTTTAGGTAGACCTGGTGCCGGTTGTTCAACTCTA 60
CDR1-4          TTGAGACCAGGGGAAGTAACTGTTGTTTTAGGTAGACCTGGTGCCGGTTGTTCAACTCTA 60
                ************************************************************

CDR1-1          TTGAAAACCATCGCTGCCCAGACATACGGGTTTCACGTAGCAAATGAGTCAATTATTACC 120
CDR1-2          TTGAAAACCATCGCTGCCCAGACATACGGGTTTCACGTAGCAAATGAGTCAATTATTACC 120
CDR1-3          TTGAAAACCATCGCTGCCCAGACATACGGGTTTCACGTAGCAAATGAGTCAATTATTACC 120
CDR1-4          TTGAAAACCATCGCTGCCCAGACATACGGGTTTCACGTAGCAAATGAGTCAATTATTACC 120
                ************************************************************

CDR1-1          TATGATGGCATGACACAAAAGGACATTGAACATCATTACCGTGGAGATGTTATATACTTT 180
CDR1-2          TATGATGGCATGACACAAAAGGACATTGAACATCATTACCGTGGAGATGTTATATACTTT 180
CDR1-3          TATGATGGCATGACACAAAAGGACATTGAACATCATTACCGTGGAGATGTTATATACTTT 180
CDR1-4          TATGATGGCATGACACAAAAGGACATTGAACATCATTACCGTGGAGATGTTATATACTTT 180
                ************************************************************

CDR1-1          GCGGAAACCGAAGTCCACATCCCACACATGACTGTTGGCCATACTTTAGAATTTGCTGCT 240
CDR1-2          GCGGAAACCGAAGTCCACATCCCACACATGACTGTTGGCCATACTTTAGAATTTGCTGCT 240
CDR1-3          GCGGAAACCGAAGTCCACATCCCACACATGACTGTTGGCCATACTTTAGAATTTGCTGCT 240
CDR1-4          GCGGAAACCGAAGTCCACATCCCACACATGACTGTTGGCCATACTTTAGAATTTGCTGCT 240
                ************************************************************

CDR1-1          CGATTAAGAACCACACAAAACAGAGGGGTTGGGATTGACAGGGAGACGTATGCAAAACTA 300
CDR1-2          CGATTAAGAACACCACAAAACAGAGGGGTTGGGATTGACAGGGAGACGTATGCAAAACTA 300
CDR1-3          CGATTAAGAACACCACAAAACAGAGGGGTTGGGATTGACAGGGAGACGTATGCAAAACTA 300
CDR1-4          CGATTAAGAACACCACAAAACAGAGGGGTTGGGATTGACAGGGAGACGTATGCAAAACTA 300
                ***********  *********************************************

CDR1-1          ATGGCTGATGCGTATATGGCTACATATGGTATATCCCACACAAGGAACACAAAAAGTTGG 360
CDR1-2          ATGGCTGATGCGTATATGGCTACATATGGTATATCCCACACAAGGAACACAAAAAGTTGG 360
CDR1-3          ATGGCTGATGCGTATATGGCTACATATGGTATATCCCACACAAGGAACACAAAAAGTTGG 360
CDR1-4          ATGGCTGATGCGTATATGGCTACATATGGTATATCCCACACAAGGAACACAAAAAGTTGG 360
                ************************************************************

CDR1-1          AATGACCTCGTACGGGGAGTTTCTGGAGGTGAGAGGAAAAGAGACATTCCTTCCCTTCCC 420
CDR1-2          AATGACCTCGTACGGGGAGTTTCTGGAGGTGAGAGGAAAAGAGACATTCCTTCCCTTCCC 420
CDR1-3          AATGACCTCGTACGGGGAGTTTCTGGAGGTGAGAGGAAAAGAGACATTCCTTCCCTTCCC 420
CDR1-4          AATGACCTCGTACGGGGAGTTTCTGGAGGTGAGAGGAAAAGAGACATTCCTTCCCTTCCC 420
                ************************************************************

CDR1-1          CTGAA 425
CDR1-2          CTGAA 430
CDR1-3          CTGAA 430
CDR1-4          CTGAA 430
                *****
```

Fig. 1: Multiple sequence alignment of 3' end portion of the CtCDR1 genes. Boxed region indicates point mutation where different amino acids were observed. Asterisks (*) indicate identical nucleotides. CDR1-1 = *C. tropicalis* ATCC 750, *C. tropicalis* resistant isolates namely CDR1-2 = Ct0557, CDR1-3 = Ct 3137, CDR1-4 = Ct0161

Clustal W CDR1 multiple sequence alignment

```
MDR1-1 TTCATGTTGGCATTCACCCTTCCTGAACGTTTCAGTAAGACTTTATTGCGTCGTAAGGCT 60
MDR1-2 TTCATGTTGGCATTCACCCTTCCTGAACGTTTCAGTAAGACTTTATTGCGTCGTAAGGCT 60
MDR1-3 TTCATGTTGGCATTCACCCTTCCTGAACGTTTCAGTAAGACTTTATTGCGTCGTAAGGCT 60
MDR1-4 TTCATGTTGGCATTCACCCTTCCTGAACGTTTCAGTAAGACTTTATTGCGTCGTAAGGCT 60
       ************************************************************

MDR1-1 CAACGTTTAAGAGCAGTTACTGGTAATGATAGAATCACTAGTGATGGTGAAGTTGAAAAT 120
MDR1-2 CAACGTTTAAGAGCAGTTACTGGTAATGATAGAATCACTAGTGATGGTGAAGTTGAAAAT 120
MDR1-3 CAACGTTTAAGAGCAGTTACTGGTAATGATAGAATCACTAGTGATGGTGAAGTTGAAAAT 120
MDR1-4 CAACGTTTAAGAGCAGTTACTGGTAATGATAGAATCACTAGTGATGGTGAAGTTGAAAAT 120
       ************************************************************

MDR1-1 TCCAAAATGACAACTCATGAATTGATTGTTGATACTTTATGGAGACCATTGGAGATTACA 180
MDR1-2 TCCAAAATGACAACTCATGAATTGATTGTTGATACTTTATGGAGACCATTGGAGATTACA 180
MDR1-3 TCCAAAATGACAACTCATGAATTGATTGTTGATACTTTATGGAGACCATTGGAGATTACA 180
MDR1-4 TCCAAAATGACAACTCATGAATTGATTGTTGATACTTTATGGAGACCATTGGAGATTACA 180
       ************************************************************

MDR1-1 ATCATGGAACCGGTTGTTTTGTTGATTGATATTTACATTGCTATGGTTTACAGTATTCTT 240
MDR1-2 ATCATGGAACCGGTTGTTTTGTTGATTGATATTTACATTGCTATGGTTTACAGTATTCTT 240
MDR1-3 ATCATGGAACCGGTTGTTTTGTTGATTGATATTTACATTGCTATGGTTTACAGTATTCTT 240
MDR1-4 ATCATGGAACCGGTTGTTTTGTTGATTGATATTTACATTGCTATGGTTTACAGTATTCTT 240
       ************************************************************

MDR1-1 TATCTTTTCTTTGAAGTTTTCCCAATTTATTTTGTTGGAGTTAGAGGATTTACTTTGGTT 300
MDR1-2 TATCTTTTCTTTGAAGTTTTCCCAATTTATTTTGTTGGAGTTAGAGGATTTACTTTGGTT 300
MDR1-3 TATCTTTTCTTTGAAGTTTTCCCAATTTATTTTGTTGGAGTTAGAGGATTTACTTTGGTT 300
MDR1-4 TATCTTTTCTTTGAAGTTTTCCCAATTTATTTTGTTGGAGTTAGAGGATTTACTTTGGTT 300
       ************************************************************

MDR1-1 GAACTTGGTACCACTTTCTTTTCCGTGTTGATTGGTATTGTTGTTGCCTGTTCTATTTTC 360
MDR1-2 GAACTTGGTACCACTTTCTTTTCCGTGTTGATTGGTATTGTTGTTGCCTGTTCTATTTAC 360
MDR1-3 GAACTTGGTACCACTTTCTTTTCCGTGTTGATTGGTATTGTTGTTGCCTGTTCTATTTAC 360
MDR1-4 GAACTTGGTACCACTTTCTTTTCCGTGTTGATTGGTATTGTTGTTGCCTGTTCTATTTAC 360
       ************************************************************

MDR1-1 TTACCTATCATCAAACGAATTTTCACTGATAGAATTCTCAGAAAAGAACAAGTTTTCCCA 420
MDR1-2 TTACCTATCATCAAACGAATTTTCACTGATAGAATTCTCAGAAAAGAACAAGTTTTCCCA 420
MDR1-3 TTACCTATCATCAAACGAATTTTCACTGATAGAATTCTCAGAAAAGAACAAGTTTTCCCA 420
MDR1-4 TTACCTATCATCAAACGAATTTTCACTGATAGAATTCTCAGAAAAGAACAAGTTTTCCCA 420
       ************************************************************

MDR1-1 GAAGTTTTCATTCCATTAGCTATAGTTGGATGGTGTTTGTTAACCGGTGGACTATTCATA 480
MDR1-2 GAAGTTTTCATTCCATTAGCTATAGTTGGATGGTGTTTGTTAACCGGTGGACTATTCATA 480
MDR1-3 GAAGTTTTCATTCCATTAGCTATAGTTGGATGGTGTTTGTTAACCGGTGGACTATTCATA 480
MDR1-4 GAAGTTTTCATTCCATTAGCTATAGTTGGATGGTGTTTGTTAACCGGTGGACTATTCATA 480
       ************************************************************

MDR1-1 TTTGGATGGTCAGCAAACTAGAACTAA 507
MDR1-2 TTTGGATGGTCAGCAAACTAGAACTAA 507
MDR1-3 TTTGGATGGTCAGCAAACTAGAACTAA 507
MDR1-4 TTTGGATGGTCAGCAAACTAGAACTAA 507
       ***************************
```

Fig. 2: Multiple sequence alignment of 5' end portion of the CtMDR1 genes. Boxed region indicates point mutation where different amino acids were observed. Asterisks (*) indicate identical nucleotides. MDR1-1 = *C. tropicalis* ATCC 750, *C. tropicalis* resistant isolates namely MDR1-2 = Ct0557, MDR1-3 = Ct 3137, MDR1-4 = Ct0161

Table 1: Primers used in this study

Primer name	Sequence (5'-3')
Ct CDR1 F	CTCTTTTCCTCTCACCTCCAGA
Ct CDR1 R	TGAGACCAGGGGAAGTAACTGT
Ct MDR1 F	TCATGTTGGATTCACCCTTC
Ct MDR1 R	TAGTTCTAGTTGCTGACCATCCAA
ERG 11 F1	ATGACGGTTTATTTRGGTCC
ERG 11 R1	GTGAAGATAGTCATTTCWGGTTG
Ca Actin F	ACCGAAGCTCCAATGAATCCAAAATCC
Ca Actin R	GTTTGGTCAATACCAGCAGCTTCCAAA

PCR amplification of CtCDR1, CtMDR1 and CtERG11: PCR amplification of Ct CDR1, Ct MDR1 and Ct ERG11 was carried out using gene specific primers, respectively (Table 1).

Sequence analysis of CDR1, MDR1 and ERG11: The CtCDR1 and CtMDR1 genes of three clinical isolates of *Candida tropicalis* which are resistant to fluconazole and the control strain *Candida tropicalis* ATCC 750 which is sensitive towards fluconazole, were amplified using specific primers and sequenced. In CDR1, point mutations were present among the resistant isolates compared to the susceptible isolate namely at C252A, A253C (T84P at amino acid level) for CDR1 and T359A for MDR1 gene (F119Y at amino acid level).

Table 2: *In vitro* fluconazole susceptibility test of *Candida tropicalis* isolates analyzed by disk diffusion method

Sample ID	Species	Mean zone of inhibition (mm)	Fluconazole resistance status
CT0557	*Candida tropicalis*	12	Resistant
CT3137	*Candida tropicalis*	12	Resistant
CT0161	*Candida tropicalis*	16	Susceptible Dose-Dependent
CT5483	*Candida tropicalis*	14	Resistant
CT4335	*Candida tropicalis*	14	Resistant
CT6337	*Candida tropicalis*	14	Resistant

The sequences were subjected to BLASTX homology search and the results indicate that the sequences had significant homology to the *C. albicans* CDR1 (78%), MDR1 (79%) and ERG11 sequences (87%). CtCDR1 sequence (Fig. 1) had not been reported yet. We report here for the first time, a partial ORF of the CtCDR1 sequence corresponding to nucleotides 1883 to 2004 of the *C. albicans* CDR1 gene. CtMDR1 sequence had been shown in Fig. 2.

As for ERG11, three clinical isolates of *Candida tropicalis* which are resistant to fluconazole and *Candida tropicalis* ATCC 750 which is sensitive towards fluconazole were also amplified and sequenced. From the

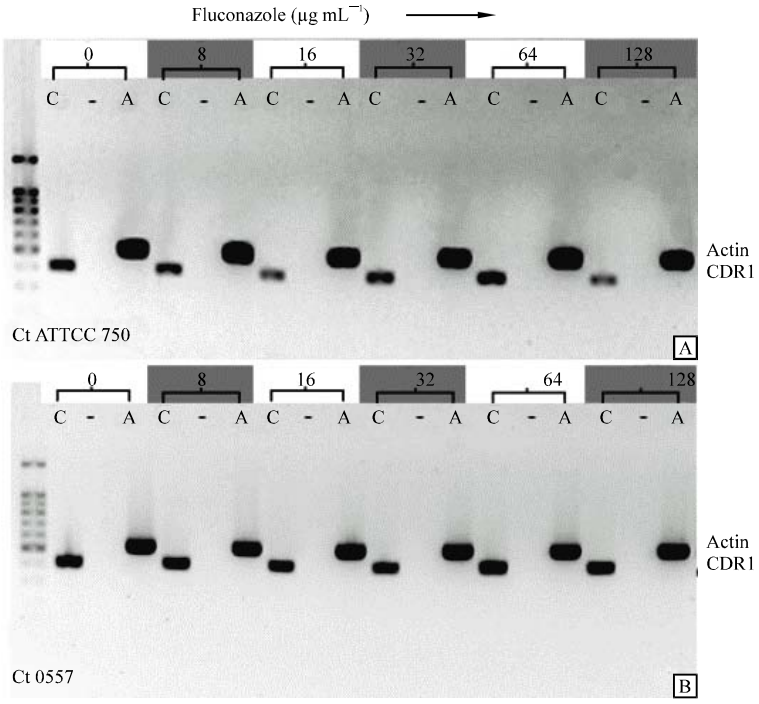

Fig. 3: RT-PCR analysis of CtCDR1 and Actin genes for drug susceptible *Candida tropicalis* CT ATCC 750 (A) and drug resistant isolate Ct0557 (B) after induction with increasing fluconazole concentrations. ['C'= CtCDR1, '-' = without MMLV reverse transcriptase, 'A' = Actin] Both isolates were subjected to culture at increasing concentrations of fluconazole. RNA was extracted and then subjected to semi-quantitative RT-PCR to access the relative gene expression of CtCDR1 gene using actin gene as housekeeping gene control

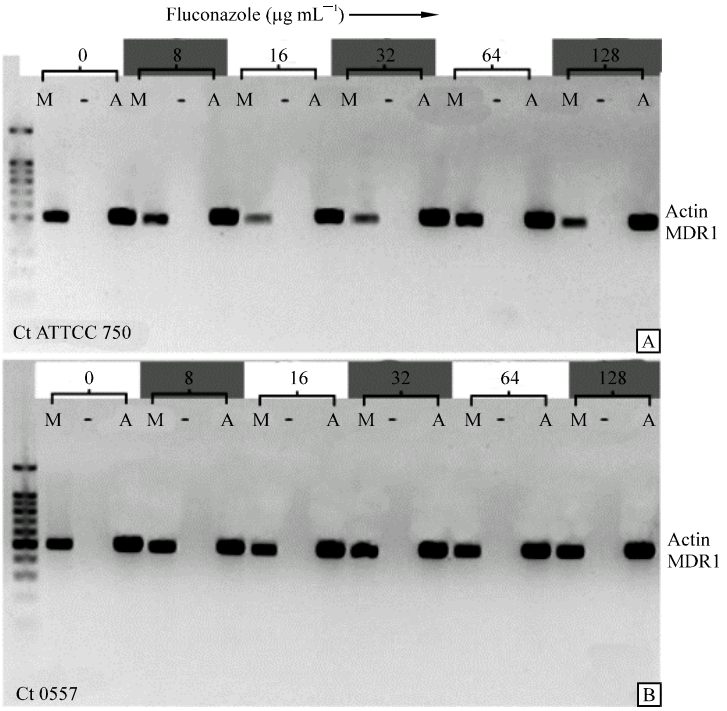

Fig. 4: RT-PCR analysis of CtMDR1 and Actin genes for drug susceptible *Candida tropicalis* CT ATCC 750 (A) and drug resistant isolate Ct0557 (B) after induction with different fluconazole concentrations. ['M' = MDR1, '-' = without MMLV reverse transcriptase, 'A '= Actin]

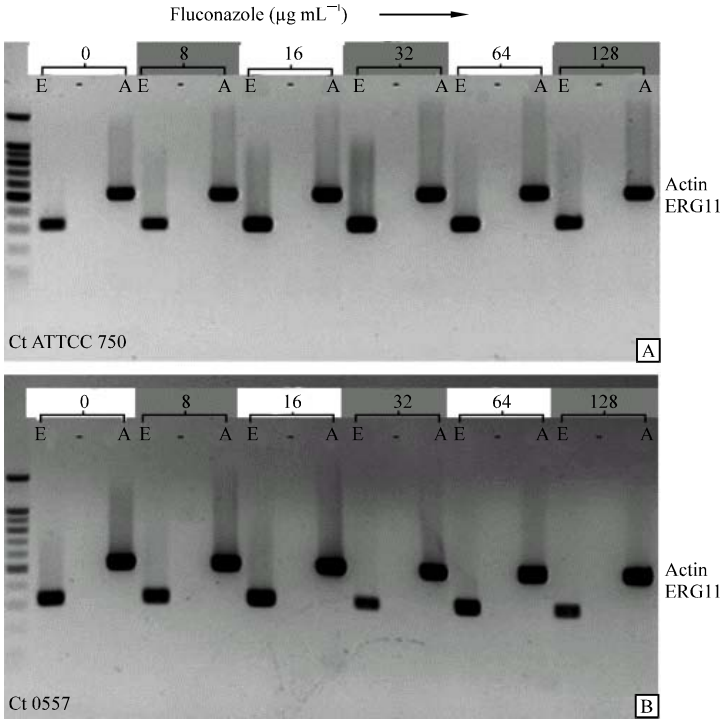

Fig. 5: RT-PCR analysis of ERG11 and Actin genes for drug susceptible *Candida tropicalis* CT ATCC 750 (A) and drug resistant isolate Ct0557 (B) after induction with different fluconazole concentrations. ['E' = ERG11, '-' = without MMLV reverse transcriptase, 'A' = Actin]

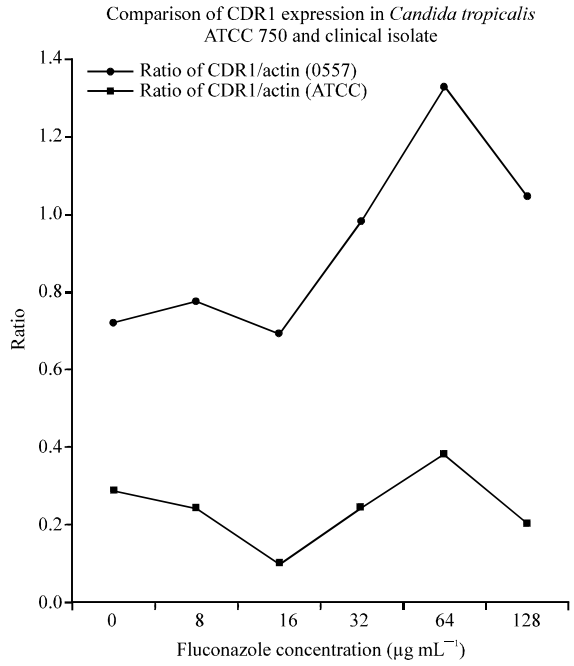

Fig. 6: Comparison of CDR1 expression in *Candida tropicalis* ATCC 750 and *Candida tropicalis* clinical isolate (0557). The relative levels of the expression were measured against the house-keeping gene, actin gene level

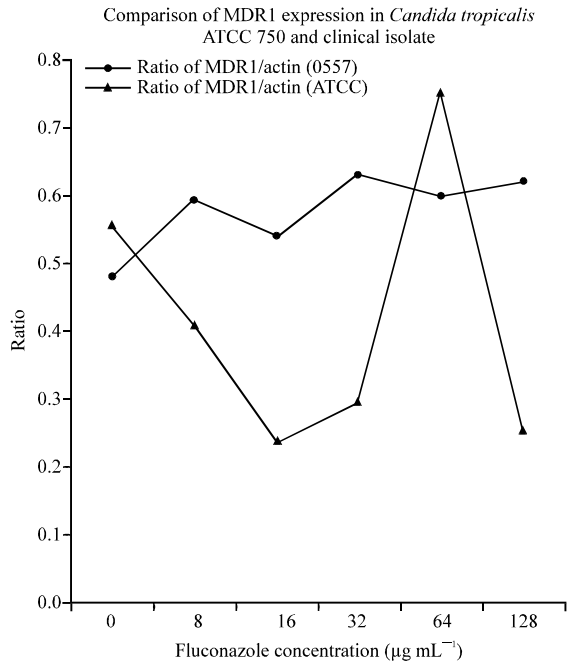

Fig. 7: Comparison of MDR1 expression in *Candida tropicalis* ATCC 750 and *Candida tropicalis* clinical isolate (0557)

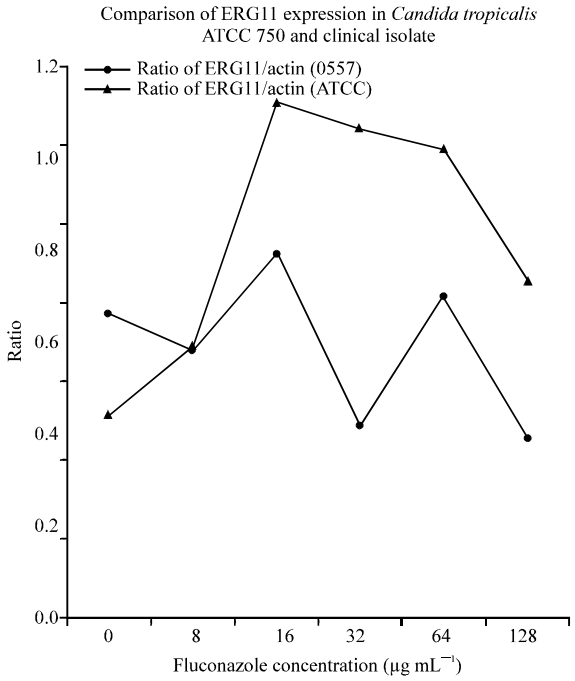

Fig. 8: Comparison of ERG11 expression in *Candida tropicalis* ATCC 750 and *Candida tropicalis* clinical isolate (0557)

sequence analysis, no point mutations were found; this included the hot spot mutation that was described in previous retrospective studies such as Y132H, R467K, G464S and others (Marichal *et al*., 1999).

Reverse transcription-PCR: Reverse Transcription- PCR was carried out using RNA from *C. tropicalis* ATCC 750 (fluconazole-susceptible isolate) and *C. tropicalis* clinical isolate 0557 (fluconazole-resistant isolate). Both isolates were induced in various fluconazole concentrations namely 128, 64, 32, 16, 8, 4, 2 and 1 µg mL^{-1}. Gel electrophoresis of the RT-PCR products for CtCDR1 and Actin genes for *Candida tropicalis* ATCC 750 and clinical isolate Ct0557 is shown in Fig. 3. RT-PCR was also performed for CtMDR1 (Fig. 4), CtERG11 (Fig. 5) and Actin for *Candida tropicalis* ATCC 750 and *Candida tropicalis* Ct0557. The amounts of mRNA of *Candida tropicalis* ATCC 750 strain which was fluconazole-susceptible were compared to *Candida tropicalis* Ct0557 which was resistant to fluconazole.

For the fluconazole-resistant *Candida tropicalis* clinical isolate, mRNA expression for CtCDR1 (Fig. 6) and CtMDR1 (Fig. 7) were higher (at 1.5, 2.2, 5.9, 3.0, 2.4, 4.1, fold) and (0.9, 1.5, 2.2, 2.1, 0.8, 2.5 fold), respectively than the *Candida tropicalis* ATCC 750 which was susceptible to fluconazole. This correlates with the degree

of resistance and was further supported by previous studies (White *et al*., 1997). An increasing trend of CDR1 expression was observed as fluconazole concentration in the medium increases (Fig. 6). Thus, this suggests that overexpression of CDR1 may be involved in *Candida* sp. resistance.

In addition to this, the ERG11 mRNA for *Candida tropicalis* ATCC 750 and *Candida tropicalis* clinical isolate showed a small and inconsistent increase in expression (Fig. 8); which ranged from 0.6 to 2.0 fold after standardization for Actin gene. In general, there was no fluconazole effect on *ERG11* mRNA level of CT ATCC strain and *Candida tropicalis* clinical isolate.

DISCUSSION

Induction of *Candida tropicalis* with fluconazole was aimed at inducing rapid transient resistance in the susceptible isolate and thus determining the involvement of CDR1 and MDR1 as prime mechanisms in rapid resistance development. Previous publications had stated that azole resistance in prolonged drug exposure may result from stable genomic alterations but rapid, transient resistance may be caused by regulation of efflux pumps on *Candida albicans* species (Marr *et al*., 1998). Moreover, another study also showed that increased mRNA levels of the efflux pump gene family and MDR1 were correlated with increased resistance (Lyons *et al*., 2000).

In CDR1 expression analysis, susceptible isolate of *Candida tropicalis* grown in 64 μg mL^{-1} fluconazole-containing medium showed an increase in CDR1 gene expression compared to an isolate grown in fluconazole-free medium. However, by increasing fluconazole concentration in the media, inconsistency of CDR1 gene expression was also observed. A likely explanation for the inconsistency is that upregulation of CDR1 expression was not efficient in low concentrations of fluconazole during 3 days induction while high concentration of fluconazole (128 μg mL^{-1}) exhibits toxicity to the *Candida* cells.

Unlike the susceptible isolate, the resistant isolate showed an increasing trend of CDR1 gene expression during the induction with the incremental of fluconazole concentration in the medium. Resistant isolates grown in fluconazole-free medium had lower CDR1 gene expression compared to isolates grown in fluconazole-containing medium. This result suggests that resistant isolates could upregulate the expression of CDR1 rapidly during encounter with fluconazole and were often associated with resistance (Marr *et al*., 1998).

Similarly, in *Candida tropicalis*, MDR1 expression levels in the resistant isolates grown in fluconazole-containing medium were higher than isolates grown in fluconazole-free medium and the expressions did not change significantly throughout the series of fluconazole concentrations. This result was further supported by a previous study describing changes in efflux pump expressions during rapid resistance in *Candida albicans* (Marr *et al*., 1998). In the susceptible isolate, MDR1 was expressed inconsistently throughout the series of fluconazole-containing medium. It has been shown in a previous study that the MDR1 mRNA was variable in expression pattern due to its short half life (Lyons *et al*., 2000).

Comparing between expression of CDR1 and MDR1 in both susceptible and resistant isolates grown throughout the fluconazole series, an alternation in overexpression of MDR1 and CDR1 was detected indicating presence of unstable phenotypes (Lopez-Ribot *et al*., 1998).

On the other hand, ERG11 gene which encodes the lanosterol demethylase, an essential enzyme in ergosterol biosynthesis pathway, has been studied extensively by many researchers with regards to its genetic alteration, expression and mechanism of resistance (Arthington *et al*., 1999). Several genetic alterations have been identified that are associated with the ERG11 gene of *C. albicans*, including point mutations in the coding region, overexpression of the gene, gene amplification (which lead to overexpression) and gene conversion or mitotic recombination (White *et al*.,1998).

However, DNA sequencing analysis conducted in this study found no point mutation in the ERG11 gene for the fluconazole - susceptible strain (CT ATCC 750) and as well as three *C. tropicalis* fluconazole-resistant isolates (Ct5483, Ct4335 and Ct6337) after comparing with the nucleotide sequence obtained from the GenBank database. This was compatible with a few studies which suggested analysis of the sequences of ERG11 and assay of cell sterol constituents revealed no differences between sensitive and resistant isolates (Marr *et al*., 1998; Lopez Ribot *et al*., 1998).

Overexpression of ERG11 has been described in several different clinical isolates by previous studies. In each case, the overexpression is not substantial. It is difficult to assess the contribution of ERG11 overexpression to a resistant phenotype, since these limited cases of overexpression have always been accompanied by other alteration associated with resistance and overexpression of genes regulating efflux pumps (White *et al*., 1998). ERG11 overexpression alone does not account for high-level azole resistance (Lopez-Ribot *et al*., 1998). However, increased mRNA levels for ERG11, MDR1 and CDR genes are associated with resistance as suggested by many studies.

Thus, more study is necessary to investigate the mechanisms that lead to the overexpression of efflux

pumps and also ERG11. Through understanding the molecular mechanism of fluconazole resistance, we hope that more effective antifungal therapies can be devised.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Science, Technology and Innovation, National Biotechnology Directorate for funding this project. A special thanks is also extended to Dr. Ng for the kind gift of precious fluconazole powder.

REFERENCES

Arthington-Skaggs, B.A., H. Jradi, T. Desai and C.J. Morrison, 1999. Quantitation of ergosterol content: Novel method for determination of fluconazole susceptibility of *Candida albicans*. J. Clin. Microbiol., 37: 3332-3337.

Barchiesi, F., D. Calabrese, D. Sanglard and D.I. Falconi *et al*., 2000. Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. Antimicrob. Agents Chemother., 44: 1578-1584.

Basu, S., H.C. Gugnani, S. Joshi and N. Gupta, 2003. Distribution of *Candida* species in different clinical sources in Delhi. India and proteinase and phospholipase activity of *Candida albican*s isolates. Rev. Iberoam Micol., 20: 137-140.

Bennett, J.E., 1990. Goodman and Gilman's the Pharmacological Basis of Therapeutics. Pergamon Press Inc, Elmsford, N.Y., pp: 1165-1181.

Feng, Q., E. Summers, B. Guo and G. Fink, 1999. Ras signaling is required for serum-induced hyphal differentiation in *Candida albican*s. J. Bacteriol., 181: 6339-6346.

Kakeya, H., Y. Miyazaki, H. Miyazaki, K. Nyswaner, B. Grimberg and J.E. Bennett, 2000. Genetic analysis of azole resistance in the Darlington strain of *Candida albicans*. Antimicrob. Agents Chemother., 44: 2985-2990.

Looi, C.Y., E.C. D'Silva, H.F. Seow, R. Rosli, K.P. Ng and P.P. Chong, 2005. Increased expression and hotpsot mutations of the multidrug efflux transporter, CDR1 in azole-resistant *Candida albicans* isolates from vaginitis patients. FEMS Microbiol., 249: 283-289.

Lopez-Ribot, J.L., R.K. McAtee, L.N. Lee, W.R. Kirkpatrick, T.C. White, D. Sanglard and T.F. Patterson, 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother., 42: 2932-2937.

Lupetti, A., R. Danesi, M. Campa, M. Del Tacca and S. Kelly, 2002. Molecular basis of resistance to azole antifungals. Trends Mol. Med., 8: 76-81.

Lyons, C.N. and T.C. White, 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. Antimicrob. Agents Chemother., 44: 2296-2303.

Marichal, P., L. Koymans, S. Willemsens, D. Bellens P. Verhasselt *et al*., 1999. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology, 10: 2701-2713.

Marr, K.A., C.N. Lyons, T.R. Rustad, R.A. Bowden and T.C. White, 1999. Rapid transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of CDR. Antimicrob. Agents Chemother. 10: 2584-2589. Erratum in: Antimicrob. Agents Chemother 1999, Rustad T., 43: 438. [corrected to Rustad TR].

Ng, K.P., M. Madasamy, T.L. Saw, A. Baki, J. He and T.S. Soo-Hoo, 1999. *Candida* biotypes isolated from clinical specimens in Malaysia. Mycopathologia, 144: 135-140.

Ng, K.P., T.L. Saw, S.L. Na and T.S. Soo-Hoo, 2001. Systemic *Candida* infection in University hospital 1997-1999: The distribution of *Candida* biotypes and antifungal susceptibility patterns. Mycopathologia, 149: 141-146.

Odds, F.C., 1993. Resistance of yeasts to azole-derivative antifungal. J. Antimicrob. Chemother., 31: 463-471.

Sanglard, D. and F.C. Odds, 2002. Resistance of *Candida* species to antifungal agents: Molecular mechanisms and clinical consequences. Lancet Infect Dis., pp: 73-85.

Sonneborn, A., D.P. Bockmuhl and J.F. Ernst, 1999. Chlamydospore formation in *Candida albicans* requires the Efg1p morphogenetic regulator. Infect. Immun., 67: 5514-5517.

White, T.C., 1997. Increased mRNA levels of ERG16, CDR and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother., 41: 1482-1487.

White, T.C., K.A. Marr and R.A. Bowden, 1998. Clinical, cellular and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev., 2: 382-402.

Zaugg, C., M. Borg-Von Zepelin, U. Reichard, D. Sanglard and M. Monod, 2001. Secreted aspartic proteinase family of *Candida tropicalis*. Infect. Immun., 69: 405-412.