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ERG6 and *ERG2* are major targets conferring reduced susceptibility to amphotericin B in clinical *Candida glabrata* isolates in Kuwait

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Running title: ERG2 and ERG6 mutations in *C. glabrata*

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ABSTRACT

Candida glabrata is intrinsically less susceptible to azoles and resistance to echinocandins and reduced susceptibility to amphotericin B has also been detected. Molecular mechanisms of reduced susceptibility (RS) to amphotericin B (AMB) were investigated in *C. glabrata* strains in Kuwait by sequence analyses of genes involved in ergosterol biosynthesis. A total of 1646 *C. glabrata* isolates were tested by Etest and results for 12 selected isolates were confirmed by reference broth microdilution. PCR-sequencing of three (*ERG2*, *ERG6* and *ERG11*) genes was performed for all RS-AMB and 5 selected wild-type *C. glabrata* isolates by using gene-specific primers. Total cell sterol content was analyzed by gas chromatography-mass spectrometry. Phylogenetic relationship among the isolates was investigated by multilocus sequence typing. Wild-type isolates contained only synonymous mutations in *ERG2*, *ERG6* or *ERG11* and total sterol content was similar to reference strains. A nonsynonymous (AGA48AAA, R48K) *ERG6* mutation was found in both RS-AMB and wild-type isolates. Four RS-AMB isolates contained novel nonsense mutations at Trp286/Tyr192/Leu341 and 2 isolates contained nonsynonymous (V126F or C198F) mutation in *ERG6* and their sterol content were consistent with *ERG6* deficiency. Two other RS-AMB isolates contained a novel nonsynonymous (G119S or G122S) *ERG2* mutation and their sterol content were consistent with *ERG2* deficiency. Of 8 RS-AMB isolates, 1 fluconazole-resistant isolate also contained nonsynonymous Y141H+L381M mutations while 7 isolates contained only synonymous mutations in *ERG11*. All isolates with *ERG6*/*ERG2/ERG11* mutations were genotypically distinct strains. Our data show that *ERG6* and *ERG2* are major targets conferring RS-AMB in clinical *C. glabrata* isolates.

Key words: *Candida glabrata*; amphotericin B; Reduced susceptibility; *ERG6*, *ERG2* and *ERG11* mutations

INTRODUCTION

Candida species are the most common cause of invasive fungal infections in seriously ill or immunocompromised hospitalized patients (1). Although *Candida albicans* is the most pathogenic species, infections by non-*albicans Candida* species have increased in recent years and are associated with high mortality rates (1-3). *Candida glabrata* is the second or third most commonly isolated yeast species causing candidemia and other invasive infections in critically ill older adult (>65 years) patients (1-3). *C. glabrata* is intrinsically less susceptible to azole antifungal drugs and causes mortality in nearly 50% subjects with invasive infections (2-4). Recent years have also witnessed increasing reports of breakthrough *C. glabrata* infections in patients receiving systemic echinocandin (micafungin) or polyene treatment (5-7).

Major antifungal drugs used for invasive *Candida* infections include triazoles (such as fluconazole, itraconazole, voriconazole, posaconazole and isavuconazole), echinocandins (such as caspofungin, anidulafungin and micafungin) and polyenes (such as amphotericin B, AMB and liposomal amphotericin B, LAMB). In contrast to azoles and echinocandins which disrupt ergosterol and glucan synthesis, respectively, polyenes were postulated, until recently, to intercalate directly with membrane ergosterol forming ion channels, which permeabilize and kill yeast cells (3, 4). More recent studies, however, have shown that AMB forms extramembranous aggregates which extract ergosterol, a central molecule in yeast physiology, from phospholipid bilayers like a sterol sponge and removal of ergosterol kills yeast cells while the contribution of ion channels is relatively minor (8). The sterol sponge model has also stipulated that simultaneous extraction of cholesterol by AMB from mammalian cells is responsible for its toxicity suggesting the possibility of separating cytocidal properties from membrane permeabilizing activities (8). Further studies have indeed resulted in the synthesis of other derivatives of AMB (such as amphotericin B methyl urea and amphotericin B amino urea) that bind ergosterol with much greater selectivity than cholesterol and thus are toxic to yeast cells but not to human cells (9).

Consistent with their mechanism of action, resistance of *Candida* spp. to triazoles and echinocandins usually develops in a stepwise manner during prolonged therapy as a result of induced changes and mutations (3, 4, 10). Since extraction of ergosterol by AMB from yeast membranes will affect all ergosterol-dependent cellular processes and membrane fluidity/hydrophobicity, evolution of amphotericin B-resistant strains is expected to be highly unlikely due to involvement of many different membrane proteins that directly bind to ergosterol as well as blocking of transport processes by several essential transport proteins due to alteration in membrane properties (11). Thus, emergence of *Candida* spp. exhibiting reduced susceptibility to AMB (RS-AMB) has generally been rare despite >50 years of clinical use. The mutations in rare RS-AMB *C. albicans* strains created defects in filamentation and tissue invasion and diverse stresses resulting in hypersensitivity to oxidative stress, febrile temperatures and neutrophil-mediated killing (12, 13). On the contrary, increasing reports of RS-AMB in *C. glabrata*, a haploid species, is a worrisome development (14-19).

Given the lower susceptibility to azoles and increasing incidence of breakthrough infections in patients on micafungin therapy, reduced susceptibility to polyenes will severely limit the choice of antifungal drugs to treat *C. glabrata* infections (3, 4). Since polyenes extract ergosterol from the cell membrane, changes in ergosterol content due to mutations in *ERG* genes involved in ergosterol biosynthesis alter susceptibility to polyenes and complete absence of ergosterol confers RS-AMB (17, 19, 20). A better understanding of the mechanisms that mediate reduced susceptibility to polyenes in *C. glabrata* is warranted. The first molecular mechanism describing RS-AMB in *C. glabrata* involved a missense (C198F) or a nonsense (at Gln332) mutation in *ERG6* encoding C24-methyl transferase that converts zymosterol to fecosterol in ergosterol biosynthesis pathway (15, 17). Deletion of *ERG11* or mutations in *ERG1*, *ERG2* and *ERG11* have also been described that are associated with RS-AMB in *C. glabrata* (18, 19, 21). Lack of *ERG6* and *ERG2* encoded enzyme activities leads to accumulation of zymosterol and fecosterol, respectively. Zymosterol and fecosterol can support fungal cell growth and absence of ergosterol in *Candida* cell membrane confers RS-AMB (15, 17, 19, 22).

Epidemiological cut-off values rather than clinical breakpoints are available for interpreting the minimum inhibitory concentrations (MICs) of AMB for *Candida* species and yeasts with MICs of $\leq$2 µg/ml were classified as wild-type (WT) while isolates with MICs >2 µg/ml were defined as non-wild-type (non-WT) (23). Antifungal drug susceptibility testing (AST) data for clinical *C. glabrata* isolates collected during 2009 to 2016 in Kuwait by Etest identified 1 isolate as non-WT while the remaining isolates were WT for AMB. However, among WT isolates, 7 strains exhibited reduced susceptibility (MIC $\geq$1 µg/ml) to AMB (RS-AMB). These isolates were used for sequence analyses of 3 (*ERG2, ERG6* and *ERG11*) ergosterol biosynthesis genes to investigate the mechanisms responsible for RS-AMB. Here we describe novel missense/nonsense mutations in *ERG6* and *ERG2* in 6 and 2 *C. glabrata* isolates, respectively, that resulted in altered sterol content and RS-AMB.

RESULTS

Characterization of study isolates and antifungal susceptibility. A total of 1646 clinical *C. glabrata* isolates were received in Mycology Reference Laboratory (MRL), Department of Microbiology, Kuwait University during 2009-2016 for species-specific identification and AST as part of routine patient care. During AST by Etest, 1 isolate was identified as non-WT while the remaining isolates were WT for AMB. However, among WT isolates, 7 strains exhibited reduced susceptibility (MIC $\geq$1 µg/ml) to AMB (RS-AMB). Thus, 8 isolates exhibited RS-AMB and were included for further studies while the remaining isolates were considered as WT. Five isolates WT for susceptibility to AMB were analyzed for comparison purpose. Two other *C. glabrata* strains isolated in 2012 that required cholesterol (or other sterols) for growth (20) were not included as their susceptibility to AMB and total cell sterol content could not be determined accurately. All isolates were identified as *C. glabrata* by Vitek 2 yeast identification system and as *C. glabrata* sensu stricto by MALDI TOF MS and by PCR amplification of rDNA (data not shown). The results were further confirmed by PCR-sequencing of the ITS region of rDNA which exhibited >99% identity with the corresponding sequences from two (ATCC90030 and CBS138) reference *C. glabrata* strains and a well-characterized clinical *C. glabrata* isolate (Kw280/06) from Kuwait analyzed previously (16), as expected. The AST data of AMB and other antifungal drugs by the reference broth microdilution method are presented in Table 1. Eight RS-AMB isolates identified by Etest also exhibited elevated MICs by broth microdilution method. Other WT isolates showed MICs $\leq$0.5 µg/ml for AMB. Only 1 RS-AMB isolate was resistant to fluconazole and also showed higher MICs for other triazoles while the remaining isolates were susceptible to triazoles. Interestingly, 4 RS-AMB isolates exhibited very low ($\leq$0.5 µg/ml) MICs for fluconazole and other triazoles. Only 1 isolate detected as WT for AMB showed reduced susceptibility to echinocandins while the remaining isolates were susceptible.

Analysis of *ERG2, ERG6* and *ERG11* gene sequences. Relative to the sequence of ERG2 protein (C-8 sterol isomerase) from *C. glabrata* reference strain CBS138 (GenBank accession number CR380958), several nonsynonymous mutations were identified in the translated ERG2 protein among clinical isolates analyzed in this study. These included I207V mutation in all study isolates, G122S mutation only in Kw844/10 and G119S mutation only in Kw3060/15 (Table 2). The replacement of glycine with serine at Gly122 in *C. glabrata* Kw844/10 and at Gly119 in *C. glabrata* Kw3060/15 apparently impaired ERG2 protein function as ergosterol was totally absent in these isolates (Table 2). Additionally, some synonymous mutations within the coding region and nucleotide insertion/deletion/substitution in non-coding region of *ERG2* were also detected in some isolates.

The results of PCR-sequencing of *ERG6* were very interesting as 3 isolates (Kw1856/15, Kw2813/15 and Kw3357/15) exhibiting RS-AMB contained a nonsense mutation and another RS-AMB isolate (Kw861/13) contained a deletion of one nucleotide (nucleotide 1021) which resulted in pre-mature termination of translated protein at codon 341 (Table 2). Although a nonsynonymous (R48K) mutation was present in some WT and some RS-AMB isolates, 2 other nonsynonymous mutations (V126F in isolate Kw96/15 and C198F in isolate Kw2516/15) were detected among 2 RS-AMB isolates only. Interestingly, 5 of 6 RS-AMB isolates cultured in 2015 contained a mutation in *ERG6*. Compared to the sequence of ERG6 protein (C24 methyl transferase) from *C. glabrata* reference strain CBS138 (GenBank accession number CR380954), some synonymous *ERG6* mutations were also detected among the study isolates and the 2 RS-AMB isolates (Kw1856/15 and Kw3357/15) with the same *ERG6* mutation (W286*) were genotypically very different strains. Consistent with PCR-sequencing results, all 6 RS-AMB isolates with *ERG6* mutations lacked ergosterol and accumulated zymosterol, the substrate for *ERG6*.

Relative to the sequence of ERG11 protein (sterol 14α-demethylase) from *C. glabrata* reference strain CBS138 (GenBank accession number CR380951), few synonymous mutations were identified in the translated ERG11 protein among all clinical isolates analyzed in this study while two nonsynonymous (Y141H + L381M) mutations were detected in one (Kw861/13) RS-AMB isolate (Table 2) that was also resistant to fluconazole (Table 1). Isolate Kw861/13 also contained *ERG6* mutation (deletion of nucleotide 1021 that resulted in creation of termination codon at Leu341), no detectable ergosterol but higher levels of lanosterol in the cell (Table 2). None of the other WT or RS-AMB isolates contained a nonsynonymous mutation in *ERG11* (Table 2). All mutations were confirmed by re-extraction of DNA from fresh cultures of *C. glabrata* isolates and PCR-sequencing of the respective *ERG* genes.

Total cell sterol composition. The total cell sterol composition of one isolate WT for AMB was very similar to the sterol composition of the reference strains ATCC90030 and Kw280/06 with ergosterol accounting for nearly 90% of total cell sterol. All 6 RS-AMB isolates with *ERG6* mutations showed marked differences in sterol content compared to the isolates WT for AMB as ergosterol was not detectable while cholesta-type sterols (including zymosterol) accumulated in the mutants (Table 3). Differences were also apparent in the abundance of various cholesta-type sterols in the 6 *ERG6* mutants. Among the 4 isolates with nonsense mutations resulting in truncated ERG6 protein, the cholesta-8,24-dienol (zymosterol) was more abundant sterol in 3 (Kw1856/15, Kw2813/15 and Kw3357/15) isolates while cholesta-5,7,24-trienol was the major accumulating sterol and cholesta-5,7,22,24-tetraenol was barely detectable in Kw861/13 (Table 3). The cholesta-5,7,24-trienol was also the major accumulating cholesta-type sterol in 2 (Kw96/15 and Kw2516/15) isolates containing nonsynonymous *ERG6* mutations, however, these isolates also accumulated cholesta-5,7,22,24-tetraenol (Table 3). Isolate Kw844/10 with a nonsynonymous (G122S) mutation and isolate Kw3060/15 with another nonsynonymous (G119S) mutation in *ERG2* also lacked ergosterol but accumulated ergosta-type (Δ8) sterols including fecosterol (ergosta-8,24(28)-dienol) (Table 3).

The genotypic relationship among the study isolates was also determined by constructing phylogenetic tree based on concatenated sequences of ITS region of rDNA, *ERG2*, *ERG6* and *ERG11* and the results are shown in FIG. 1. Both (Kw844/10 and Kw3060/15) isolates with a missense mutation in *ERG2* and the two (Kw96/15 and Kw2516/15) isolates with a missense mutation in *ERG6* were genotypically distinct strains. Furthermore, all four (Kw861/13, Kw1856/15, Kw2813/15 and Kw3357/15) isolates with a nonsense mutation in *ERG6* were also unique strains (FIG. 1). Fingerprinting of selected isolates performed with 6-loci-based MLST also showed that most of the isolates analyzed were unique strains (Table 2).

DISCUSSION

Invasive fungal infections are difficult to treat due to availability of only few classes of antifungal agents. Among invasive *Candida* infections, *C. glabrata* infections are more common among elderly (≥65-year-old) hospitalized patients who usually have several debilitating conditions and so these infections are generally associated with higher mortality (24-26). Although resistance of *Candida* spp. to azole antifungal drugs is common and resistance to echinocandins also appeared soon after their introduction in clinical practice nearly 15 years ago, RS-AMB is uncommon despite >50 years of clinical use as sequestering of ergosterol by extramembranous aggregates of amphotericin B deprives phospholipid membranes of a sterol essential for many different aspects of yeast physiology (8, 9, 11). In diploid *C. albicans*, RS-AMB is rare due to fitness trade-offs which abrogate fungal virulence (9, 10). However, there have been several reports of RS-AMB in haploid *C. glabrata* and breakthrough infections in patients receiving systemic LAMB is a worrisome development for effective management of invasive *C. glabrata* infections (7, 14, 15, 17-19).

Although molecular mechanisms involved in azole resistance are well recognized, those involved in conferring RS-AMB are poorly defined (3, 4, 24, 26). Results from this study on 8 *C. glabrata* isolates with RS-AMB showed that 4 isolates contained a nonsense mutation at codons Tyr192 or Trp286 or Leu341 resulting in premature translational termination of ERG6 transcripts. The mutant cells accumulated cholesta-type sterols (including cholesta-8,24-dienol or zymosterol) indicating that the truncated ERG6 proteins were inactive. Since polyenes act as a sterol sponge and extract ergosterol from phospholipid bilayer (8, 9), absence of ergosterol in yeast cell membrane was likely responsible for RS-AMB in these isolates. Nonsense mutations at codons Tyr192 or Trp286 or Leu341 in ERG6 described in this study are novel mutations described for the first time. Premature termination of ERG6 protein due to another nonsense mutation at codon Gln332 was recently described in a *C. glabrata* isolate with RS-AMB, the mutant cells lacked ergosterol but accumulated ergosterol pathway intermediates and wild-type properties were restored in complementation studies with a wild-type copy of the *ERG6* gene (17). The total cell sterol analysis of our isolates also showed differences in the accumulation of individual cholesta-type sterols in the 4 mutant strains. The mutation in isolate Kw861/13 introduced the stop codon only 32 amino acids before the C-terminal end of C-24 sterol methyltransferase encoded by ERG6 while nonsense mutations in the other 3 (Kw1856/15, Kw2813/15 and Kw3357/15) isolates resulted in shortening of the ERG6 protein by 87 or more amino acids at the C-terminal end. Previous studies have shown the presence of two conserved domains in methyltransferases located between amino acid positions 134 to 222 and the second domain between 231 and the C-terminal end (27, 28). The second domain was only 32 amino acids shorter in isolate Kw861/13. As *ERG6* encoded enzyme catalyzes the conversion of zymosterol into fecosterol by C-24 methylation, isolate Kw861/13, like the other 3 isolates lacked fecosterol but unlike the other 3 isolates contained low level of 14-methyl fecosterol suggesting modification of C-24 sterol methyltransferase activity in Kw861/13. Furthermore, isolate Kw861/13, in addition to exhibiting RS-AMB, also exhibited resistance to fluconazole and other azoles, contained two nonsynonymous mutations (Y141H + L381M) in *ERG11* and accumulated lanosterol suggesting loss of sterol 14α-demethylase activity which conferred resistance to azoles. Fluconazole-resistant clinical *C. glabrata* isolates usually contain gain-of-function mutations in the gene encoding transcription factor *CgPDR1* which results in upregulation of drug efflux transporters encoded by *CgCDR1* and *CgCDR2* genes and to a lesser extent, *CgCNQ2* (3, 4). Only few studies have reported azole resistance conferring mutations in *ERG11*, the main target of azoles in *C. albicans* and some other non-*albicans Candida* species (3, 4, 18, 29). A clinical *C. glabrata* isolate (CG156) with a nonsynonymous (G315D) mutation in substrate recognition site of *ERG11* has been described previously that was resistant to triazoles (fluconazole and voriconazole) and AMB and accumulated lanosterol due to complete loss of sterol 14α-demethylase activity (18). The mutated CG156 Erg11p failed to complement the function of a doxycycline-regulatable *Saccharomyces cerevisiae erg11* strain while wild-type *C. glabrata* Erg11p fully complemented, supporting of role of ERG11 mutation in conferring resistance to fluconazole. Another recent study from Iran has also suggested the involvement of another nonsynonymous mutation (G236V) in *ERG11* as the main mechanism conferring resistance to azoles in a clinical *C. glabrata* isolate (R1) even though several other nonsynonymous mutations were also detected in this and other isolates (29). However, the role of G236V mutation in altering the function of *ERG11* was speculated solely based on homology modelling studies but was not confirmed by sterol analysis or by gene replacement studies (29). It will be interesting to see if resistance conferring *ERG11* mutations are also found in other fluconazole-resistant *C. glabrata* isolates in Kuwait.

Two other RS-AMB isolates contained two different nonsynonymous mutations in *ERG6*. Of these V126F mutation in Kw96/15 is a novel mutation while C198F mutation found in isolate Kw2516/15 has been described previously in an RS-AMB *C. glabrata* isolate (isolate no. 21229) that lacked ergosterol in cell membrane, exhibited pseudohyphal growth, accumulated late sterol intermediates and overexpressed genes encoding enzymes involved in late steps of ergosterol biosynthesis pathway (15). Complementation studies with a wild-type copy of *ERG6* gene restored WT pattern of AMB susceptibility for isolate 21229 demonstrating the role of ERG6 protein in conferring resistance to polyenes (15). Consistent with these observations, isolate Kw96/15 and Kw2516/15 also lacked ergosterol and accumulated cholesta-type sterols. As seen above with different nonsense mutations, isolate Kw96/15 and Kw2516/15 also showed variations in the accumulation of individual intermediates with zymosterol (cholesta-8,24-dienol) and cholesta-5,7,22,24-tetraenol levels varying by nearly 2-fold and accumulation of detectable levels of ergosta-5,7-dienol in isolate Kw96/15 but not in isolate Kw2516/15. The findings are consistent with observations that the enzymes encoded by various *ERG* genes and involved in ergosterol biosynthesis pathway may act on similar substrates leading to formation of several sterol intermediates (15, 22, 30). Furthermore, similar to isolate 21229 with ERG6 mutation (15), and an *erg1* mutant of *C. glabrata* (31), both Kw96/15 and Kw2516/15 also exhibited increased susceptibility to azoles. Similarly, 2 isolates (Kw1856/15 with W286* and Kw2813/15 with Y192*) with nonsense mutations also exhibited increased susceptibility to azoles while another isolate (Kw3357/15 with W286*) exhibited slightly higher MIC value for fluconazole. It has been suggested that isolate 21229 with C198F mutation in ERG6 becomes more susceptible to azoles due to presence of Δ5,7-dienols (cholesta-type sterols) which maintain the cell viability but do not completely replace ergosterol functionally (15). Another consequence of altered sterol composition in the mutant cells is disturbance of protein trafficking preventing targeting of ABC transporters (CgCDR1p and CgCDR2p) to the plasma membrane and the decreased efflux capacity likely contributes to increased sensitivity to azoles (15, 32).

Although a nonsynonymous mutation (I207V) was found in *ERG2* in all 13 *C. glabrata* isolates from Kuwait, this alteration represents genetic polymorphism not associated with RS-AMB since it has also been described previously in *C. glabrata* isolates WT for AMB as well as in isolates with RS-AMB (19). However, 2 RS-AMB (Kw844/10 and Kw3060/15) *C. glabrata* isolates from Kuwait contained novel nonsynonymous mutations (G122S and G119S) in *ERG2*. Both isolates (Kw844/10 and Kw3060/15) lacked ergosterol and accumulated ergosta-type sterols including fecosterol (Δ8 sterols). Two RS-AMB *C. glabrata* isolates containing nonsynonymous mutations at *ERG2* codon Thr121 (T121I and T121V) have been described recently (19). Both *ERG2* mutant isolates (CG852 and CG872) in that study (19) also lacked ergosterol and accumulated Δ8 sterols indicating impaired function of ERG2 protein. Thr121 in ERG2 protein is likely involved in the binding of sterol substrate as the corresponding amino acid (Thr119) in *S. cerevisiae* is involved in sterol Δ8-Δ7 isomerization (19, 33). Since Gly119 and Gly122 are located on either side of the Thr121, they may be critical in maintaining the structure of the active site and the extended region (codons 119 to 122) may constitute the ERG2 protein region conferring RS-AMB.

An intriguing observation of our study pertains to the fact that despite complete absence of ergosterol from mutant *C. glabrata* cells, the increase in MIC values for AMB were only modest as they were still categorized within WT category for susceptibility to AMB. These observations and the presence of significant amounts of various ergosta-type and cholesta-type sterols in mutant *C. glabrata* cells suggest that at least some of these sterols can also maintain fungal membrane fluidity and are also sensitive to removal by the extramembranous AMB sponge. It will be interesting to see how these mutant strains will respond to other derivatives of AMB that have recently been synthesized to overcome the problem of toxicity of AMB to mammalian cells (9, 34, 35).

Fingerprinting of the isolates carried out by sequence comparisons of ITS region of rDNA together with ERG2, ERG6 and ERG11 sequences showed that all study isolates were genotypically distinct strains. The 6-loci-based MLST carried out on selected RS-AMB isolates also showed genetic variations among the isolates as they usually belonged to different STs. These findings suggest that RS-AMB in *C. glabrata* isolates in Kuwait is not clonal.

In conclusion, 8 RS-AMB *C. glabrata* strains were isolated in Kuwait and 6 of these 8 isolates were obtained in 2015. Six isolates contained a nonsense or nonsynonymous mutation in ERG6 while 2 isolates contained a nonsynonymous mutation in ERG2 and the total cell sterol content were consistent with ERG6 or ERG2 deficiency. Fingerprinting studies showed that RS-AMB in *C. glabrata* isolates in Kuwait was not clonal. The data show that *ERG6* and *ERG2* are major targets conferring RS-AMB in clinical *C. glabrata* isolates.

MATERIALS AND METHODS

Yeast strains, culture conditions and identification. Reference strains of *Candida glabrata* (ATCC90030, CBS138 and a well characterized clinical isolate, Kw280/06) were used. During 2009 to 2016, 1646 clinical *C. glabrata* isolates were received in the Mycology Reference Laboratory (MRL), Department of Microbiology, Faculty of Medicine, Kuwait University. The isolates were cultured in BACTEC Plus blood culture bottles (Beckton Dickinson, Sparks, MD, USA) from various clinical specimens in different hospitals across Kuwait after obtaining informed verbal consent as part of routine patient care and diagnostic work-up. The isolates were initially subcultured at MRL on Sabouraud dextrose agar (SDA) for species-specific identification and antifungal susceptibility testing (AST) as part of routine patient care (16). Few isolates failed to grow on SDA upon subculturing and required addition of cholesterol to SDA for their growth, as described in detail previously (20).

Phenotypic identification of the isolates was initially performed by Vitek 2 yeast identification system (bioMérieux, Marcy-l'Etoile, France). The isolates were also identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) as described previously (36). The genomic DNA from reference strains and clinical isolates was extracted by using Gentra Puregene Yeast DNA extraction kit (Qiagen, Hilden, Germany) according to kit instructions or by the rapid method using Chelex-100, as described previously (37). Species-specific identification was performed by PCR amplification of rDNA as described previously (38). The complete ITS region was also amplified by using ITS1 and ITS4 primers and the amplicons were sequenced by using ITS1FS, ITS2, ITS3 and ITS4RS primers, as described previously (39, 40).

Antifungal susceptibility testing. The antifungal susceptibility testing (AST) of *C. glabrata* isolates to AMB was initially carried out on SDA and minimum inhibitory concentrations (MICs) were determined by the Etest procedure (AB Biodisk, Solna, Sweden) according to manufacturer's instructions and as described previously (41). The isolates were also tested against different antifungal drugs by the CLSI reference broth microdilution method as described previously (42, 43) and susceptibility breakpoints for different antifungal agents were those described earlier (23, 43, 44). *C. glabrata* ATCC90030 was used as reference strain during AST.

***ERG* genes sequencing.** The complete coding sequence and the flanking 5' and 3' untranslated regions of *ERG2, ERG6* and *ERG11* genes for all study isolates were amplified from genomic DNA by using gene-specific primers. The *ERG2* gene was amplified by using CgERG2F (5'-CTAAACGAGCTCGTAATTCTA-3') and CgERG2R (5'-GCCTTAGAGTCTATCCTTGAA-3') primers by using the PCR amplification reaction and cycling conditions, as described previously (37, 39). The amplicons were purified by using PCR product purification kit (Qiagen, Hilden, Germany) according to kit instructions. Both strands of purified amplicons were sequenced by using forward (CgERG2FS1, 5'-GCTCGTAATTCTATCGGTTTGA-3'; CgERG2FS2, 5'-TTGCAATGGTGTACTTGCCAA-3'; CgERG2FS3, 5'-GGTATGACCCATCATCTACA-3') and reverse (CgERG2RS1, 5'-GATTCTGTAGAGGCACTAGCA-3'; CgERG2RS2, 5'-CCTTGAGCCAATTCTAGTGCGA-3'; CgERG2RS3, 5'-CCTTAGAGTCTATCCTTGAATTA-3') primers with BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Austin, TX, USA) and ABI 3130*xl* Genetic

Analyzer by following manufacturer's instructions (Applied Biosystems) and as described previously (20, 37). The complete *ERG2* sequences of ~1060 bp were assembled and were compared with the corresponding sequences from reference *C. glabrata* strain CBS138 by using the program Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

The *ERG6* gene was amplified and sequenced as two overlapping fragments. The N-terminal fragment was amplified by using CgERG6F1 (5'-GATTTTCTCGTTTCGCCGAGAA-3') and CgERG6R1 (5'-GATGATGTTACAGCCGGTGAA-3') primers while the C-terminal fragment was amplified by using CgERG6F2 (5'-ACGAACAGTACTTGGCATACA-3') and CgERG6R2 (5'-CATGTGGAATGAATTCAAGTGA-3') primers and PCR amplification reaction and cycling conditions, as described previously (37, 39). The amplicons were purified and both strands were sequenced by using the gene-specific primers and the sequencing protocol described previously (20, 37). The sequencing primers for the N-terminal fragment included forward (CgERG6FS1, 5'-CTCGTTTCGCCGAGAATTGTTA-3' or CgERG6FS2, 5'-CAGTTTATTGTGCTCTTGACG-3') and reverse (CgERG6RS1, 5'-TCGGGAGAATTTCAATTCCTT-3' or CgERG6RS2, 5'-GATGTTACAGCCGGTGAATCT-3') primers. The primers for the C-terminal fragment included forward (CgERG6FS3, 5'-GAACAGTACTTGGCATACATGG-3' or CgERG6FS4, 5'-TTTGAAGAACGTCGGTTTCG-3') and reverse (CgERG6RS3, 5'-GTACTTCCATTCTCCGGTCAA-3' or CgERG6RS4, 5'-GTGGAATGAATTCAAGTGAACA-3') primers. The complete *ERG6* sequences of ~1840 bp were assembled and were compared with the corresponding sequences from reference *C. glabrata* strain CBS138 by using the program Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

The *ERG11* was amplified and sequenced from all study isolates as described previously (20). The complete *ERG11* sequences of ~1920 bp were assembled and were compared with the corresponding sequences from reference *C. glabrata* strain CBS138 by using Clustal omega.

Fingerprinting of *C. glabrata* isolates. The gene sequences were analyzed individually or nucleotide sequences of *ERG2*, *ERG6* and *ERG11* genes together with ITS region of rDNA were included in the combined analysis. Multiple sequence alignments of concatenated sequence data were performed with Clustalw muscle (https://www.ebi.ac.uk/Tools/msa/muscle/) and phylogenetic analysis was performed with Molecular Evolutionary Genetic Analysis (MEGA) version 6 software. The phylogenetic tree was constructed using the Neighbor-joining method with Kimura 2-parameter model. The DNA sequence data from *C. glabrata* strain CBS138 was used as reference and the robustness of tree branches was assessed by bootstrap analysis with 1,000 replicates. The genotypic relationship among selected *C. glabrata* isolates was also studied by 6 (*FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1* and *URA3*) loci-based multilocus sequence typing (MLST) scheme, as described previously (45). The DNA sequences for each gene fragment were used for allelic profile and the combined data set were used to determine the sequence types (STs) (45). Since a curated MLST database is not available for *C. glabrata*, new STs from Kuwait were given an arbitrary number (STKN1, STKN2 etc).

Sterol analysis. The total cell sterol content of *C. glabrata* isolates was determined by inoculating 15-ml volumes of MOPS-buffered (0.165 M MOPS) RPMI medium (pH 7.0) with single colonies and the cultures were grown for 24 h at 37°C. Cells were harvested by centrifugation and washed three times with sterile water before sterol extraction. Non-saponifiable lipids were extracted by using alcoholic KOH as described previously (46). Samples were dried in a vacuum centrifuge (Heto) and were derivatized with trimethylsilane (TMS) by the addition of 100 µl of 90% N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA)/10% TMS (Sigma), 200 µl anhydrous pyridine (Sigma) and heating for 2 h at 80^{o}C. The TMS-derivatized sterols were analyzed by using gas chromatography-mass spectrometry (GC-MS) (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer, Thermo Scientific, Loughborough, UK) and identified with reference to retention times and fragmentation spectra for known standards (18, 46). The GC/MS data files were analyzed by using Xcalibur software (Thermo Scientific) to determine sterol profiles for study isolates and for integrated peak areas. The results of three replicates from each sample were used to calculate the mean percentage ± standard deviation for each sterol.

Patient samples. The clinical specimens which yielded the *C. glabrata* isolates described in this study were obtained from different patients after obtaining informed verbal consent as part of routine patient care and diagnostic work-up. The isolates were also analyzed in the Mycology Reference Laboratory in the Department of Microbiology, Faculty of Medicine, Kuwait University, for identification and antifungal susceptibility testing as part of routine patient care, and the results from deidentified samples are described in this paper.

Accession numbers. The DNA sequence data reported in this study have been submitted to GenBank under accession numbers LS398111 to LS398136, LS398591 to LS398603 and LS399273 to LS399285.

REFERENCES

1. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016. Executive summary: clinical practice guideline for the management of candidiasis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis 62: 409-417.
2. Guinea J. 2014. Global trends in the distribution of *Candida* species causing candidemia. Clin Microbiol Infect 20 Suppl 6: 5-10.
3. Sanguinetti M, Posteraro B, Lass-Flörl C. 2015. Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. Mycoses 58 Suppl 2: 2-13.
4. Kołaczkowska A, Kołaczkowski M. 2016. Drug resistance mechanisms and their regulation in non-albicans Candida species. J Antimicrob Chemother 71: 1438-1450.
5. Lewis JS 2nd, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. 2013. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. Antimicrob Agents Chemother 57: 4559-4561.
6. Saraya T, Tanabe K, Araki K, Yonetani S, Makino H, Watanabe T, Tsujimoto N, Takata S, Kurai D, Ishii H, Miyazaki Y, Takizawa H, Goto H. 2014. Breakthrough invasive *Candida glabrata* in patients on micafungin: a novel FKS gene conversion correlated with sequential elevation of MIC. J Clin Microbiol 52: 2709-2712.
7. Kimura M, Araoka H, Yamamoto H, Nakamura S, Nagi M, Yamagoe S, Miyazaki Y, Ogura S, Mitsuki T, Yuasa M, Kaji D, Kageyama K, Nishida A, Taya Y, Shimazu H, Ishiwata K,

Takagi S, Yamamoto G, Asano-Mori Y, Uchida N, Wake A, Taniguchi S, Yoneyama A. 2017. Clinical and microbiological characteristics of breakthrough candidemia in allogeneic hematopoietic stem cell transplant recipients in a Japanese hospital. Antimicrob Agents Chemother 61: e01791-16.

8. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Maryum N, Wang S, Uno BE, Wildeman EL, Gonen T, Rienstra CM, Burke MD. 2014. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol. 10: 400-406.
9. Davis SA, Vincent BM, Endo MM, Whitesell L, Marchillo K, Andes DR, Lindquist S, Burke MD. 2015. Nontoxic antimicrobials that evade drug resistance. Nat Chem Biol 11: 481-487.
10. Khan Z, Ahmad S, Mokaddas E, Meis JF, Joseph L, Abdullah A, Vayalil S. 2018. Development of echinocandin resistance in *Candida tropicalis* following short-term exposure to caspofungin for empiric therapy. Antimicrob Agents Chemother 62: e01926-17.
11. te Welscher YM, van Leeuwen MR, de Kruijff B, Dijksterhuis J, Breukink E. 2012. Polyene antibiotic that inhibits membrane transport proteins. Proc Natl Acad Sci U S A 109: 11156-11159.
12. Xu D, Jiang B, Ketela T, Lemieux S, Veillette K, Martel N, Davison J, Sillaots S, Trosok S, Bachewich C, Bussey H, Youngman P, Roemer T. 2007. Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. PLoS Pathog 3: e92.
13. Vincent BM, Lancaster AK, Scherz-Shouval R, Whitesell L, Lindquist S. 2013. Fitness trade-offs restrict the evolution of resistance to amphotericin B. PLoS Biol 11: e1001692.

14. Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen D. 2006. Amphotericn B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. Clin Infect Dis 42: 938-944.
15. Vandeputte P, Tronchin G, Bergès T, Hennequin C, Chabasse D, Bouchara JP. 2007. Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of *Candida glabrata* with pseudohyphal growth. Antimicrob Agents Chemother 51: 982-990.
16. Khan ZU, Ahmad S, Al-Obaid I, Al-Sweih NA, Joseph L, Farhat D. 2008. Emergence of resistance to amphotericin B and triazoles in *Candida glabrata* vaginal isolates in a case of recurrent vaginitis. J Chemother 20: 488-491.
17. Vandeputte P, Tronchin G, Larcher G, Ernoult E, Bergès T, Chabasse D, Bouchara JP. 2008. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of *Candida glabrata*. Antimicrob Agents Chemother 52: 3701-3709.
18. Hull CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL. 2012. Facultative sterol uptake in an ergosterol-deficient clinical isolate of *Candida glabrata* harboring a missense mutation in ERG11 and exhibiting cross-resistance to azoles and amphotericin B. Antimicrob Agents Chemother 56: 4223-4232.
19. Hull CM, Bader O, Parker JE, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL. 2012. Two clinical isolates of *Candida glabrata* exhibiting reduced sensitivity to amphotericin B both harbor mutations in ERG2. Antimicrob Agents Chemother 56: 6417-6421.
20. Khan Z, Ahmad S, Joseph L, Al-Obaid K. 2014. Isolation of cholesterol-dependent, multidrug-resistant *Candida glabrata* strains from blood cultures of a candidemia patient in Kuwait. BMC Infect Dis 14: 188.

21. Geber A, Hitchcock CA, Swartz JE, Pullen FS, Marsden KE, Kwon-Chung KJ, Bennett JE. 1995. Deletion of the *Candida glabrata* ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob Agents Chemother 39: 2708-2717.
22. Young LY, Hull CM, Heitman J. 2003. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in *Candida lusitaniae*. Antimicrob Agents Chemother 47: 2717-2724.
23. Pfaller MA, Diekema DJ. 2012. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J Clin Microbiol. 50: 2846-2856.
24. Glöckner A, Cornely OA. 2015. *Candida glabrata*: unique features and challenges in the clinical management of invasive infections. Mycoses 58: 445-450.
25. Gabaldón T, Carreté L. 2016. The birth of a deadly yeast: tracing the evolutionary emergence of virulence traits in *Candida glabrata*. FEMS Yeast Res 16: fov110.
26. Arendrup MC, Patterson TF. 2017. Multidrug-resistant *Candida*: epidemiology, molecular mechanisms, and treatment. J Infect Dis 216 (suppl-3): S445-S451.
27. Bouvier-Nave P, Husselstein T, Benveniste P. 1998. Two families of sterol methyltransferases are involved in the first and the second methylation steps of plant sterol biosynthesis. Eur J Biochem 256: 88-96.
28. Jensen-Pergakes KL, Kennedy MA, Lees ND, Barbuch R, Koegel C, Bard M. 1998. Sequencing, disruption, and characterization of the *Candida albicans* sterol methyltransferase (ERG6) gene: drug susceptibility studies in *erg6* mutants. Antimicrob Agents Chemother 42: 1160-1167.

29. Nabili M, Abdollahi Gohar A, Badali H, Mohammadi R, Moazeni M. 2016. Amino acid substitutions in Erg11p of azole-resistant *Candida glabrata*: Possible effective substitutions and homology modelling. J Glob Antimicrob Resist 5: 42-46.

30. Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M. 1989. The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol 9: 3447-3456.

31. Tsai HF, Bard M, Izumikawa K, Krol AA, Sturm AM, Culbertson NT, Pierson CA, Bennett JE. 2004. *Candida glabrata erg1* mutant with increased sensitivity to azoles and to low oxygen tension. Antimicrob Agents Chemother 48: 2483-2489.

32. Umebayashi K, Nakano A. 2003. Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. J Cell Biol 161: 1117-1131.

33. Rahier A, Pierre S, Riveill G, Karst F. 2008. Identification of essential amino acid residues in a sterol 8,7-isomerase from Zea mays reveals functional homology and diversity with the isomerases of animal and fungal origin. Biochem J 414: 247-259.

34. Wilcock BC, Endo MM, Uno BE, Burke MD. 2013. C2'-OH of amphotericin B plays an important role in binding the primary sterol of human cells but not yeast cells. J Am Chem Soc 135: 8488-8491.

35. Davis SA, Della Ripa LA, Hu L, Cioffi AG, Pogorelov TV, Rienstra CM, Burke MD. 2015. C3-OH of amphotericin B plays an important role in ion conductance. J Am Chem Soc 137: 15102-15104.

36. Jamal WY, Ahmad S, Khan ZU, Rotimi VO. 2014. Comparative evaluation of two matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems for the identification of clinically significant yeasts. Int J Infect Dis 26: 167-170.

37. Asadzadeh M, Ahmad S, Hagen F, Meis JF, Al-Sweih N, Khan Z. 2015. Simple, low-cost detection of *Candida parapsilosis* complex isolates and molecular fingerprinting of *Candida orthopsilosis* strains in Kuwait by ITS region sequencing and amplified fragment length polymorphism analysis. PLoS One 10: e0142880.

38. Ahmad S, Mustafa AS, Khan Z, Al-Rifaiy AI, Khan ZU. 2004. PCR-enzyme immunoassay of rDNA in the diagnosis of candidemia and comparison with amplicon detection by agarose gel electrophoresis. Int J Med Microbiol 294: 45-51.

39. Khan ZU, Ahmad S, Mokaddas E, Chandy R, Cano J, Guarro J. 2008. *Actinomucor elegans* var. *kuwaitiensis* isolated from the wound of a diabetic patient. Antonie van Leeuwoenhoek 94: 343-352.

40. Khan ZU, Ahmad S, Hagen F, Fell JW, Kowshik T, Chandy R, Boekhout T. 2010. *Cryptococcus randhawai* sp. nov., a novel anamorphic basidiomycetous yeast isolated from tree trunk hollow of *Ficus religiosa* (peepal tree) from New Delhi, India. Antonie Van Leeuwenhoek 97: 253-259.

41. Asadzadeh M, Al-Sweih NA, Ahmad S, Khan ZU. 2008. Antifungal susceptibility of clinical *Candida parapsilosis* isolates in Kuwait. Mycoses 51: 318-323.

42. Clinical and Laboratory Standards Institute (CLSI). 2008. M27-A3. Reference method for broth dilution antifungal susceptibility testing of yeasts, Third edition. Wayne, PA: CLSI.

43. Clinical and Laboratory Standards Institute. 2012. Fourth informational supplement M27-S4. 4th. Wayne, PA: 2012. Reference method for broth dilution antifungal susceptibility testing of yeasts. Clinical Laboratory Standards Institute, Wayne, PA.

44. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016. Clinical practice

guidelines for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 62: e1-50.

45. Dodgson AR, Pujol C, Denning DW, Soll DR, Fox AJ. 2003. Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. J Clin Microbiol 41: 5709-5717.
46. Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE. 1995. Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8,24(28)-dien-3β, 6α-diol. Biochem Biophys Res Commun 207: 910-915.

TABLE 1 Source of isolation and minimum inhibitory concentration (MIC) values of 13 *C. glabrata* isolates for

(AMB) by Etest and for AMB and other antifungal drugs by broth microdilution method

Isolate no.	Clinical specimen	AMB MIC (µg/ml) by Etest	MIC values (µg/ml) by broth microdilution method for						
			AMB	FLC	ITC	VOR	POS	ISA	AN
ATCC90030[a]	N. A.	0.094	0.5	4	0.125	0.125	0.25	0.125	0.01
Kw280/06[a]	Vaginal swab	0.047	0.5	16	0.5	0.25	0.25	0.5	0.03
Kw600/09	Wound swab	0.032	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D
Kw164/15	Urine	0.38	0.25	16	0.25	0.25	0.25	0.125	0.5
Kw383/15	Ascitic fluid	0.19	0.5	2	0.125	0.031	0.125	0.031	0.01
Kw590/15	Sputum	0.003	0.25	16	0.25	0.25	0.25	0.125	0.01
Kw1421/16	Bone	0.25	0.5	1	0.063	0.031	0.063	0.016	0.03
Kw844/10	Urine	1.5	1	2	0.125	0.031	0.125	0.031	0.03
Kw861/13	ET aspirate	1	1	>64	0.5	2	0.25	0.5	0.01
Kw96/15	Urine	2	1	0.5	0.063	<0.016	0.063	<0.016	0.03
Kw1856/15	Urine	1.5	1	0.25	0.125	<0.016	0.125	<0.016	0.01
Kw2516/15	Urine	1.5	1	0.5	0.063	0.031	0.125	0.016	0.01
Kw2813/15	Urine	4	1	0.25	0.031	<0.016	0.031	<0.016	0.01
Kw3060/15	Wound swab	1.5	1	1	0.063	<0.016	0.063	<0.016	0.03
Kw3357/15	Urine	2	1	2	0.5	0.125	0.25	0.016	0.03

[a]*C. glabrata* strains ATCC90030 and Kw280/06 were used as reference strains

ET aspirate, endotracheal aspirate; MIC, minimum inhibitory concentration; AMB, amphotericin B; FLC, fluconazole; ITC, itraconazo

POS, posaconazole; ISA, isavuconazole; ANI, anidulafungin; MIC, micafungin; N. A., not available; N. D., not done

TABLE 2 Susceptibility to amphotericin B (AMB), mutations detected in *ERG2, ERG6* and *ERG11* genes, finge ERG2-ERG6-ERG11 sequence-based profile and by 6-loci-based sequence types and content of ergosterol, feco lanosterol as a percentage of total cell sterol in 13 *C. glabrata* isolates

Isolate no.	Clinical specimen	Susceptibility to AMB[a]	Non-synonymous mutations detected in			ITS-*ERG2*-*ERG6*-ERG11-based profile	MLST-based STs	% of total cel	
			ERG2	*ERG6*	*ERG11*			Ergosterol	Fecostero
ATCC90030	N. A.	Wild-type	Reference[b]	Reference[b]	Reference[b]	Reference[a]	N. D.	**93.0 ± 0.1**	1.0 ± 0.0
Kw280/06	Vaginal swab	Wild-type	I207V	R48K	None	Unique	ST-KN1	**84.8 ± 2.8**	2.0 ± 0.3
Kw600/09	Wound swab	Wild-type	I207V	None	None	Unique	N. D.	N. D.	N. D.
Kw164/15	Urine	Wild-type	I207V	None	None	Unique	N. D.	N. D.	N. D.
Kw383/15	Ascitic fluid	Wild-type	I207V	R48K	None	Unique	N. D.	N. D.	N. D.
Kw590/15	Sputum	Wild-type	I207V	R48K	None	Unique	N. D.	N. D.	N. D.
Kw1421/16	Bone	Wild-type	I207V	None	None	Unique	N. D.	**89.5 ± 7.2**	2.1 ± 1.6
Kw844/10	Urine	RS-AMB	I207V + G122S	None	None	Unique	N. D.	0	**14.9 ± 2.**
Kw861/13	ET aspirate	RS-AMB	I207V	Δ Nt. 1021, L341*	Y141H + L381M	Unique	ST-KN2	0	0
Kw96/15	Urine	RS-AMB	I207V	V126F	None	Unique	ST-46	0	0
Kw1856/15	Urine	RS-AMB	I207V	W286*	None	Unique	ST-46	0	0
Kw2516/15	Urine	RS-AMB	I207V	R48K + C198F	None	Unique	ST-KN3	0	0
Kw2813/15	Urine	RS-AMB	I207V	Y192*	None	Unique	ST-KN4	0	0
Kw3060/15	Wound swab	RS-AMB	I207V + G119S	None	None	Unique	N. D.	0	**18.9 ± 2.**
Kw3357/15	Urine	RS-AMB	I207V	W286*	None	Unique	N. D.	0	0

[a] *C. glabrata* isolates with MIC values ≤ 0.5 μg/ml were classified as wild-type for AMB while those with MIC values ≥ 1 μg/ml were reduced susceptibility to AMB (RS-AMB)

[b] *C. glabrata* strain ATCC90030 was used as reference strain for susceptibility to amphotericin B (AMB) and strain CBS138 was used *ERG* gene sequencing

*Denotes a nonsense mutation; ST, sequence type; ST-KN, new sequence type detected in Kuwait; N. D., not done

Sterol values (mean of 3 replicates with standard deviation) are percentage of total sterols and values >5% of total cell sterol are shown

TABLE 3 Total *C. glabrata* cell sterol composition in an isolate (Kw1421/16) wild-type for AMB and 7 isolates

susceptibility to AMB

Type of sterol	Percentage of each sterol in the total cell sterol composition of *C. glabrata* iso						
	ATCC90030[a]	Kw280/06[a]	Kw1421/16	Kw844/10	Kw861/13	Kw96/15	Kw1856/15	Kw2516/15
Ergosta-5,8,22,24-tetraenol	0.9 ± 0.1	3.4 ± 1.2	0.7 ± 0.1	1.5 + 0.0				
Unknown sterol					1.4 ± 0.1	1.4 ± 0.0	0.9 ± 0.1	1.2 ± 0.0
Ergosta-5,8,22-trienol	0.9 ± 0.0	1.0 ± 0.0	0.4 ± 0.3	**25.8 ± 0.3**				
Ergosta-8,22-dienol				**7.0 ± 0.7**				
Ergosta-5,8,24-trienol				**8.2 ± 0.5**				
Ergosta-5,8-dienol				**6.0 ± 0.4**				
Cholesta-5,8,24-trienol					**5.1 ± 0.3**	**9.0 ± 0.3**	**5.2 ± 0.5**	**9.5 ± 0.5**
Zymosterol (Cholesta-8,24-dienol)		2.5 ± 0.6			**21.4 ± 0.8**	**14.5 ± 0.5**	**42.5 ± 1.3**	**35.5 ± 0.6**
Cholesta-5,7,24-trienol	1.1 ± 0.0		1.6 ± 1.3		**56.0 ± 0.4**	**40.9 ± 0.5**	**34.0 ± 0.2**	**36.9 ± 0.7**
Ergosterol	**93.0 ± 0.1**	**84.8 ± 2.8**	**89.5 ± 7.2**					
Cholesta-7,24-dienol					1.3 ± 0.5	1.0 ± 0.1	1.9 ± 0.3	0.8 ± 0.0
Cholesta-5,7,22,24-tetraenol					0.1 ± 0.2	**26.7 ± 0.9**	**11.5 ± 0.8**	**13.6 ± 1.6**
14-Methyl fecosterol					1.0 ± 0.0			
Fecosterol (Ergosta-8,24(28)-dienol)	1.0 ± 0.0	2.0 ± 0.3	2.1 ± 1.6	**14.9 ± 2.2**				
Ergosta-8-enol				**30.9 ± 1.3**				
Ergosta-5,7-dienol	1.3 ± 0.2	1.8 ± 0.1	2.2 ± 1.7		0.3 ± 0.3	**5.9 ± 0.3**		
Episterol (Ergosta-7,24(28)-dienol)	0.2 ± 0.0	0.7 ± 0.1	1.2 ± 0.9					
Unknown sterol				2.1 + 0.3				0.7 ± 0.2
14-Methyl ergosta-8,24(28)-dien-3,6-diol								
Lanosterol	1.3 ± 0.1	3.2 ± 0.3	2.1 ± 1.6	2.5 ± 0.1	**11.6 ± 1.0**	0.6 ± 0.1	3.3 ± 0.3	1.3 ± 0.2
4,4-Dimethyl cholesta-8,24-dienol	0.2 ± 0.1	0.6 ± 0.4	0.2 ± 0.2	1.1 + 0.1	1.9 ± 0.2		0.8 ± 0.2	0.4 ± 0.1

[a] *C. glabrata* strains ATCC90030 and Kw280/06 were used as reference strain for determining total cell sterol composition; AMB, amp

Sterol values (mean of 3 replicates with standard deviation) are percentage of total sterols and values >5% of total cell sterol are shown

FIGURE legend:

FIG. 1. Neighbor-joining phylogenetic tree based on combined sequence data for ITS region of rDNA, *ERG2, ERG6* and *ERG11* genes from 13 clinical *C. glabrata* isolates from Kuwait together with reference *C. glabrata* strains CBS138 and Kw280/06. The numbers on the nodes branches are bootstrap frequencies.

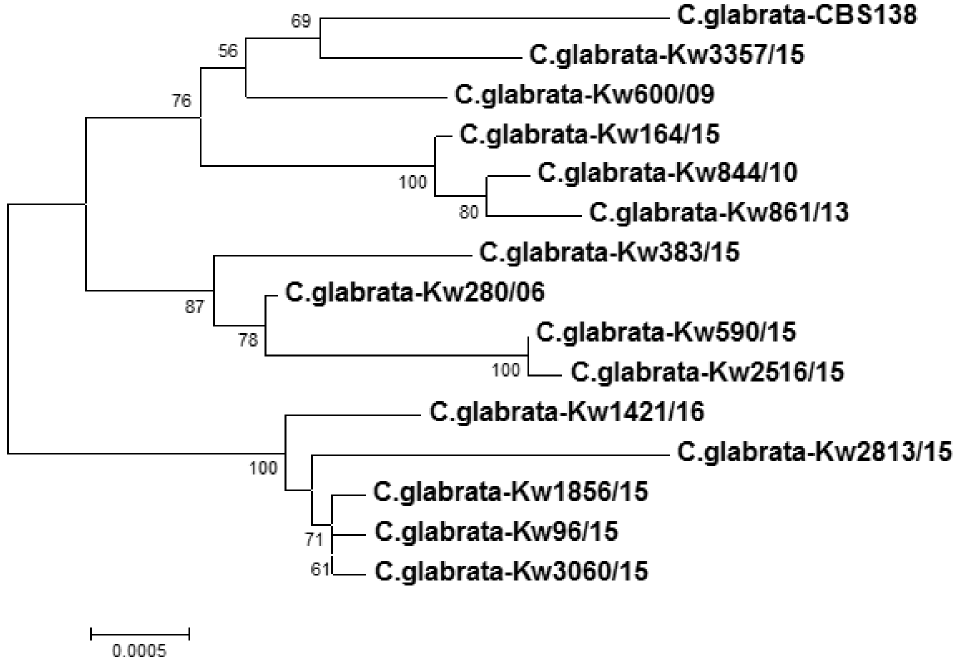
69
C.glabrata-CBS138
56
C.glabrata-Kw3357/15
76
C.glabrata-Kw600/09
C.glabrata-Kw164/15
100
C.glabrata-Kw844/10
80
C.glabrata-Kw861/13
C.glabrata-Kw383/15
87
C.glabrata-Kw280/06
78
C.glabrata-Kw590/15
100
C.glabrata-Kw2516/15
C.glabrata-Kw1421/16
100
C.glabrata-Kw2813/15
C.glabrata-Kw1856/15
71
C.glabrata-Kw96/15
61
C.glabrata-Kw3060/15
0.0005

FIG. 1